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# **Fructose and the Maillard reaction**

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**Thesis submitted for the degree of Doctor of Philosophy in  
the discipline of Biophysics**

**Open University  
Oxford Research Unit**

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**To Yvonne and Robert Liggins**

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Jason Liggins, 1996.

## Abstract

It is widely accepted that the Maillard reaction, specifically the development of Advanced Glycation End-products (AGEs) *in vivo*, is linked to the pathogenesis of diabetic secondary complications. The same diseases that occur in old age are similarly thought to develop as a function of the accumulation of AGEs. This thesis presents an *in vitro* investigation into glycation by fructose (fructation) and discusses the potential for *in vivo* fructation. The contribution of *in vivo* fructation to degenerative diseases is unknown, largely because assays for glycation underestimate, or do not detect, fructation (Ahmed N & Furth A.J. 1992, Clin. Chem. 38, 1301-1303).

*In vitro* radiolabelling illustrates that incorporation of fructose into protein, through the Maillard reaction, is more swift than glucose. The development of AGE-fluorescence reflects the incorporation of the two sugars, i.e. higher upon fructation.

A novel colorimetric assay for glycation (including fructation), based on the reaction of 2,4-dinitrophenylhydrazine (DNPH) with protein-bound carbonyl groups, is presented. The DNPH assay detects compounds that are intermediates in post-Amadori, or post-Heyns reactions, and precede development of fluorescence. The production of the protein-bound carbonyl intermediates is largely dependent on the presence of lipid and free metal. The same group of compounds are probably the principle site of action of aminoguanidine, which blocks the production of AGEs.

An *in vitro* model is presented, to show how protein glycated in one part of the body, can long after maintenance of euglycaemia, become covalently cross-linked to a second protein. Lysozyme was glycated with low concentrations of fructose,

or using short periods of fructation, and the free fructose removed. The fructated lysozyme was subsequently incubated in sugar-free buffer with native  $\beta$ -lactoglobulin and produced a 32kD heterodimer of the two covalently cross-linked proteins.

## Abbreviations

A	Absorbance
AGE	Advanced glycation end-product
AP	Amadori product
ATP	Adenosine triphosphate
$\beta$ D	$\beta$ -lactoglobulin homodimer
$\beta$ M	$\beta$ -lactoglobulin monomer
BSA	Bovine serum albumin
c	Concentration
C3	Compliment protein 3
CAT	Catalase
CCBB	Colloidal Coomassie brilliant blue
CMhL	Carboxymethylhydroxylysine
CML	Carboxymethyllysine
CV	Coefficient of variation
3-DOG	3-Deoxyglucosone
3-DOF	3-Deoxyfructose
DCCT	Diabetes control and complications trial
DNPH	2, 4-dinitrophenylhydrazine
DTPA	Diethylenetriaminepentaacetic acid
DTT	Dithiothreitol
$\epsilon$	Extinction coefficient
em.	Emission
ex.	Excitation
FFI	Furoyl-furanyl-imidazole
H	heterodimer
Hb	Haemoglobin

HFCS	High fructose corn syrup
HPLC	High performance liquid chromatography
HSA	Human serum albumin
IDDM	Insulin dependent diabetes mellitus
Ig	Immunoglobulin
l	Light path length
LD	lysozyme homodimer
LM	lysozyme monomer
M	Molar
MCO	Metal catalysed oxidation
$\mu$ M	micromolar
mM	millimolar
mg	milligrams
mins	minutes
ml	millilitres
OGTT	Oral gucose tolerance test
PAGE	Polyacrylamide gel electrophoresis
PDA	Piperazine diacrylamide
RNase	Ribonuclease A
RSA	Rat serum albumin
SDS	Sodium dodecyl sulphate
SE	standard error of the mean
SOD	Super oxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine

TES	N-tris[hydroxymethyl]methyl-2-aminoethane-sulphonic acid; 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethane sulphonic acid
TFA	Trifluoroacetic acid
UHQ	Ultra high quality
UV	Ultra violet
v/v	proportion of volume per volume
w/v	proportion of weight per volume



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# 1. Introduction

The work described in this thesis concerns the Maillard reaction as it occurs *in vivo* with particular emphasis on the monosaccharide fructose. The Maillard reaction is a non-enzymatic reaction between the carbonyl group of reducing sugars and primary amines eventually producing Advanced Glycation End-products or AGEs. There are two halves to the Maillard reaction (see Fig. 1.1.). I have used the term glycation to define the initial half of the reaction, i.e. both the formation of the Schiffs base and the subsequent rearrangement to Amadori product, if the hexose was an aldehyde, or Heyns product if the hexose was a ketone. The chemistry of glycation is well understood it is the latter half of the Maillard reaction, that I have termed post-Amadori or post-Heyns reactions, that is poorly understood. The lack of knowledge of the post-Amadori or post-Heyns reactions, both at theoretical and practical levels is because of the large number of variations of possible post-Amadori or post-Heyns reactions and their products. This thesis describes the results and conclusions of a series of experiments designed to elucidate post-Amadori or post-Heyns reactions.

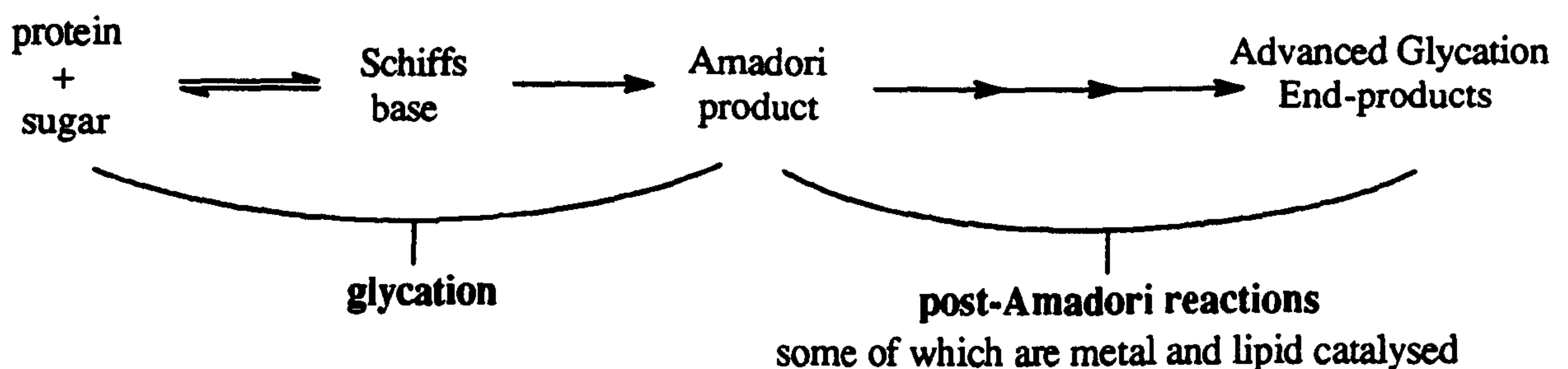


Fig. 1.1. Schematic outline of the Maillard reaction. Please note that the diagram has been simplified and the name Amadori could be substituted with Heyns to allow further definition.

Glycation occurs on primary amino groups, i.e. terminal  $\alpha$ -amino groups or the  $\epsilon$ -amino group of lysine; this makes it distinct from enzymatic glycosylation which occurs on the side groups of serine, threonine, asparagine, hydroxylysine and hydroxyproline residues. Glycation is non-enzymatic, it is a chemical reaction with a rate that is dependent on the concentration of the substrates, the temperature and the pH [133, 1, 2]. *In vitro*, temperature is an important consideration of the Maillard reaction because it affects it in two ways; the first concerns the general rule of doubling the rate of a chemical reaction for every 10°C rise. The second consideration concerns the proportion of the sugar that is in the reactive acyclic (aldehyde or ketone) form of the monosaccharide, glucose for example has a four fold increase in its aldehyde form between 20°C and 60°C [3]. To avoid problems with varying rate of reaction due to different temperatures used in different experiments the work presented in this thesis (unless otherwise stated) was carried out at 37°C, the physiological temperature of humans.

The time scale of discovery of the various parts of the Maillard reaction is very long. Schiff in 1900 first described the equilibrium between sugars, primary amines and what we know now as Schiff's base [4]. The reaction as a whole is now known as the Maillard reaction because in 1912, 1916 and 1917 Maillard showed that, upon heating, reducing sugars reacted at various rates with different amino acids to produce darkly coloured compounds [reviewed in 2]. It was Amadori in 1931 who showed that the Schiff's base of aldehydes rearranged to form what we know now as Amadori product, see figure 1.2. [5]. Ramsey *et al*, 1933 showed that proteins produced similar products to amino acids upon heating with reducing sugars, but it was Mohammed *et al*, 1949 who showed it was the free amino groups of the proteins that took part in the reaction [6 and 7]. In 1967 Heyns *et al*

reported a similar rearrangement reaction, to Amadori, of Schiff's base of ketones, to form what are now known as Heyns products [8].

The Maillard reaction is very important to the food industry because it affects nutrient availability, flavour, aroma and colour of cooked or stored foods [reviewed in 212]. During storage of foods with a high sugar content, such as milk (bovine milk contains an average of 0.13M lactose), free amino acids react with the sugar and because these products are indigestible it reduces the amino acids available to the nutrient pool [207, 9 and 212]. The reaction of sugars with free amino acids during cooking is important for the production of the coloured and volatile compounds responsible for the colour, aroma and flavour of foods, for example the aroma and colour of bread and meat or the bitterness of coffee [212, 10]. Some of the Maillard products produced by cooking protein-rich foods, especially meat and fish are very potent mutagens and carcinogens [212 and 11].



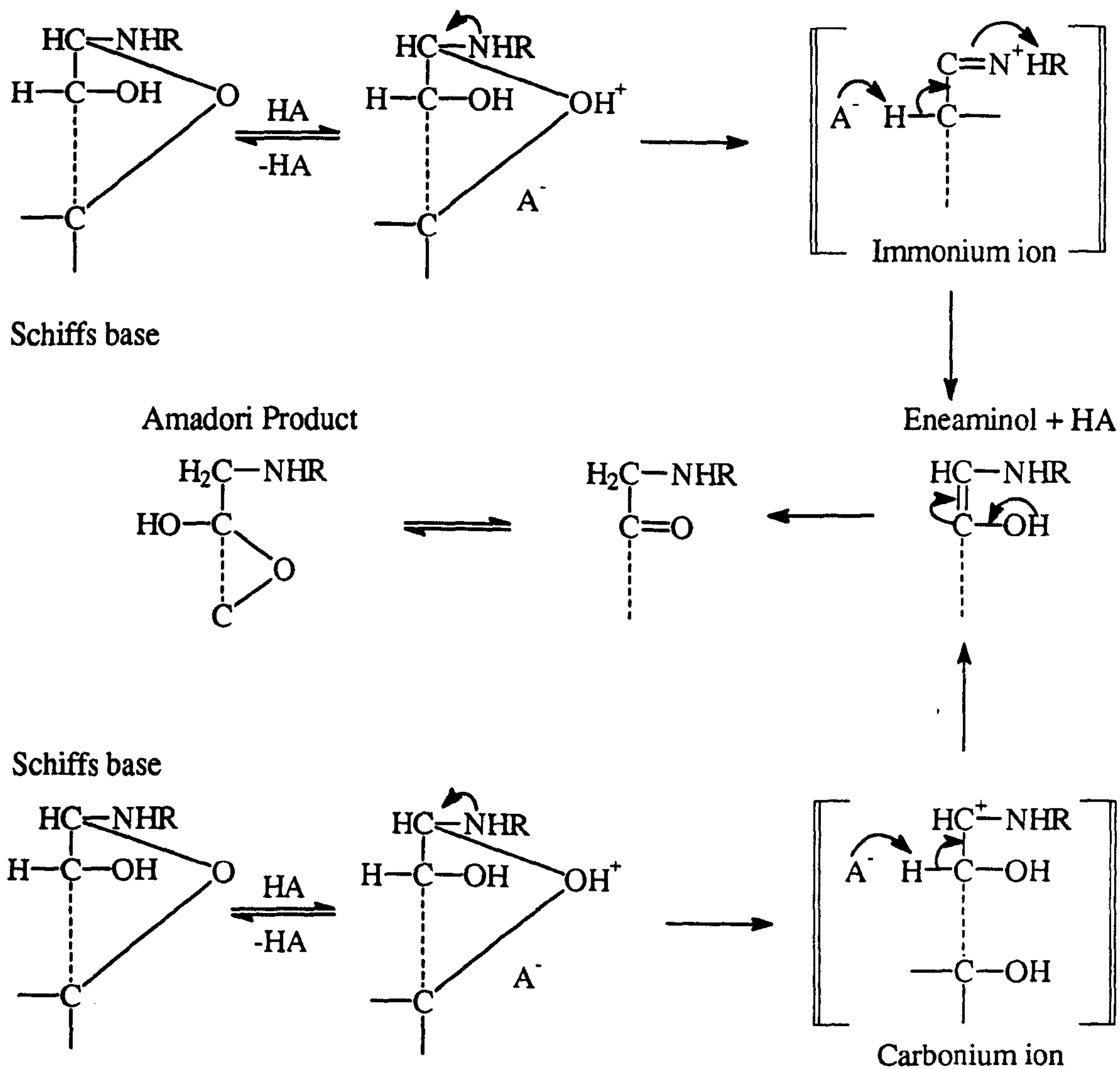


Fig. 1.2. Two possible routes for Amadori rearrangement, taken from Figs. 2 and 3 of the review by Gottschalk (1966) [13].

## 1.1. The Maillard reaction *in vivo*

It was Maillard in 1912 who first postulated upon the significance of the reaction occurring *in vivo*. Glucose is present in circulation normally at a fasting rest concentration of about 5mM, but after a meal rises as a function of the amount of carbohydrate ingested. The hormones glucagon and insulin maintain euglycaemia; glucagon prevents hypoglycaemia by mobilising glycogen while insulin prevents hyperglycaemia by increasing uptake and utilisation of glucose. High loads of glucose result in hyperglycaemia because it takes time for the control by insulin to take place; the degree and duration of hyperglycaemia experienced is a measure of glycaemic control and can be tested by an oral glucose tolerance test (OGTT). An OGTT consists of drinking a solution of 50g glucose (75g in USA) while at rest, after an overnight fast and subsequent measurement of the blood glucose concentration at various times up to two hours after ingestion; the test is used as the basis for diagnosis of diabetes mellitus (which is hereafter referred to as diabetes).

Figure 1.1.1. shows the results of a typical OGTT on a range of people as they age; the lowest line on the graph is typical of a 20 year old individual and shows a small rise in blood glucose, due to the oral load, that returns to normal within about two hours. As people age their sensitivity to insulin decreases and even though they produce more insulin, upon an OGTT their blood glucose rises higher and takes longer to return to basal levels. The top line on the graph is typical of a mild age-onset diabetic who responds poorly to the insulin that they do produce, i.e. the hyperglycaemia rises quite high and takes longer than 2 hours to return to basal levels. Type 1 diabetics also exhibit the same sort of curve as type 2 but produce much higher hyperglycaemia (20-40mM), due to the lack of insulin rather

than insensitivity to it. The gradual increase in insensitivity to insulin with age is why for many years type 2 diabetes was called age onset diabetes; up to 50% of the population in the USA is clinically or sub-clinically diabetic at age 70 [16].

This thesis is concerned with the long term changes caused by the slight hyperglycaemia ever present in conventionally treated diabetics. If type 1 diabetes goes untreated, the impaired uptake of glucose, caused by the lack of insulin, accelerates triacylglycerol hydrolysis, fatty acid oxidation and ketone body formation. Ketones are acidic so ketosis (the build up of ketones) puts a strain on the buffering capacity of the blood and kidneys. The kidneys try to control ketosis by excreting protons which increases the blood pH, but also causes a loss of sodium and potassium ions along with inorganic phosphate and water; producing overall, the classic symptoms of diabetes, i.e. thirst and decreased blood volume. Ketosis is a serious condition that can lead to coma and death, so type 1 diabetics control their blood glucose by administration of insulin.

Insulin is the dominant hormone over its antagonist glucagon, thus administration of too much insulin can suppress the blood glucose level below normal (called hypoglycaemia). Hypoglycaemia is dangerous because the brain is dependent on glucose for its energy supply and this condition can result in various the symptoms:- mood swings, sweating, change in pallor, a strong bounding pulse, shallow breathing and if left untreated will lead to coma, brain damage and even death. The constant daily danger of hypoglycaemia has meant that conventional insulin treatment has tried to produce a glucose concentration that was slightly above normal but not so high as to cause ketosis; this of course leads to a higher rate of glycation than occurs in a non-diabetic.

Measurement of the amount of glycated protein (as a percentage of the total amount of that protein) indicates the degree of glycaemic control a diabetic has



had over the life-span or half-life of that protein. The methods commonly used in routine clinical laboratories for assessment of glycaemic control are glycated haemoglobin and the serum fructosamine assay, the various methods used to detect glycation are discussed in greater detail in section 1.4. The Maillard reaction is however important *in vivo* for another reason; the build up of AGEs is directly proportional to the development of diabetic secondary complications and also to normal ageing.



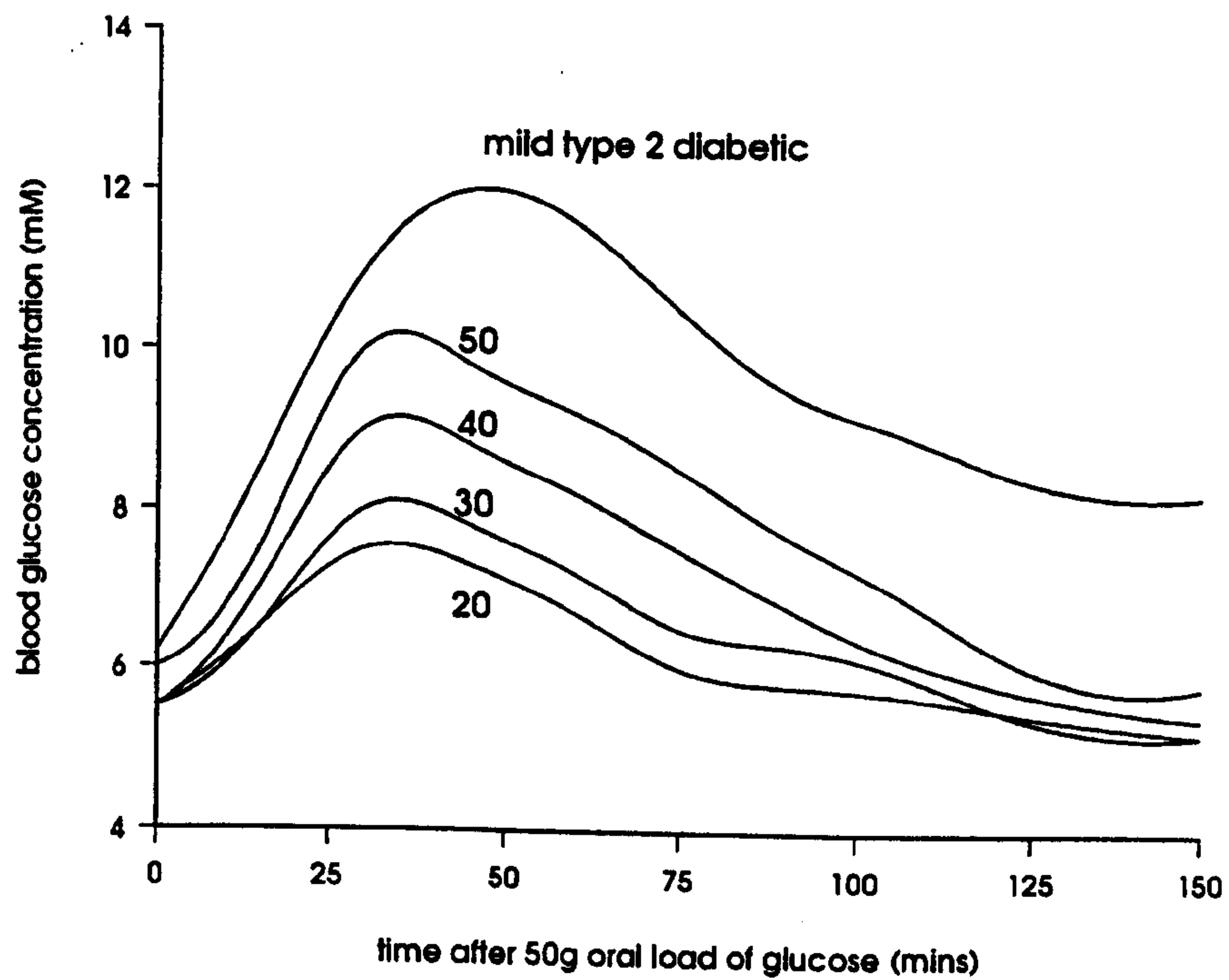


Fig. 1.1.1. Typical results of an oral glucose tolerance test (OGTT) on a range of people. The top curve is that of a mild type 2 diabetic followed by (in decreasing order) 50, 40, 30 and 20 year old individuals.

## **1.2. Diabetic secondary complications**

Insulin stimulates enhanced uptake of glucose, a lack of or insensitivity to insulin results in hyperglycaemia which in turn leads to an enhanced rate of glycation. The body controls the basal glycation, which occurs in everyone as an unfortunate side effect of using glucose as an energy source, by protein turnover. There is evidence of enhanced rates of such protein turnover stimulated by macrophages that detect the modified protein [17 and 18]. For many years hyperglycaemia was known to be a major risk factor for the development of diabetic secondary complications, but a connection between direct cause and effect was unproven and contentious. The diabetes control and complications trial (DCCT) in America has conclusively shown that hyperglycaemia is indeed the major risk factor in development of diabetic secondary complications [19]. It is the development of AGEs that is intimately connected with diabetic secondary complications; they have been shown, by a variety of methods, to increase in atherosclerosis, nephropathy, neuropathy and retinopathy as a function of age and to increase during the progression of these same diseases as diabetic secondary complications [20, 21, 22, 23, 24, 25, 26, 27, 28, 29 and 30].

Excellent reviews of diabetic secondary complications are by Yki-Järvinen (1992) and Nathan (1993) and only brief descriptions are necessary here [22 and 31]. A number of the diabetic secondary complications described here involve thickening of endothelial basement membranes. The thickening of the basement membranes is caused by tissue remodelling by the surrounding cells; such tissue remodelling could be initiated by macrophages responding to immunoglobulins trapped within the membrane or by direct recognition of an AGE modified protein [17, 18 and 215]. Interestingly the endothelial cells of murine coronary vasculature

endocytose labelled-AGE-albumin, that was in general circulation, and transcytose it to the abluminal surface, i.e. the basement membrane side. The accumulation of such labelled-AGE-albumin was reported to occur principally in the liver, lung, kidney, intestine and heart of mice into which it had been intravenously injected. The receptor for the AGE (RAGE) was reported to also occur in smooth muscle, mesangial cells and some neurons [215].

**Retinopathy** is a form of blindness caused by the formation of new capillaries over the retina that prevent light from reaching it. New capillaries are formed because the existing capillaries are blocked by the thickening of their basement membrane and increased platelet aggregation which restricts the supply of oxygen to the retina [22 and 32].

**Corneal clouding and lens cataracts** are due to a loss of order of the collagen fibrils or crystallin proteins respectively. The loss of regular protein packing is either due to AGE-cross-links between the proteins or an alternative theory holds that a build up of sorbitol causes osmotic pressure to disrupt the packing; the production of sorbitol is discussed on page 42 *et sequa* [33].

**Nephropathy** is a gradual loss of kidney function due to thickening of the glomerular and tubular basement membranes and a decrease in their heparan sulphate proteoglycan content. The kidneys gradually lose their ability to act as an ultrafilter and cannot retain protein, this is partly overcome but also exacerbated by increased glomerular filtration rate and renal plasma flow. The disease's main histological feature is proteinuria [22, 21 and 34].

**Neuropathy** or impaired nerve conduction is associated with depletion of myoinositol and decreased sodium/potassium ATPase activity, which means the nerve takes longer than normal to recover from an impulse. Activation of the



sorbitol pathway during hyperglycaemia depletes myoinositol and causes the characteristic changes in neuropathy [27, 33, 22 and 34].

**Macrovascular disease** that includes arteriosclerosis, atherosclerosis and thrombosis are due to AGE-cross-linking, thickening of circulatory vessel basement membrane, deposition and modification of serum proteins (e.g. lipoproteins) into the membrane, altered blood viscosity and impaired endothelial dependent relaxation [21, 35, 36, 37, 22 and 23].

**Secondary infections** occur at a higher incidence in people who suffer from hyperglycaemia because of impaired neutrophil adhesion, chemotaxis and phagocytosis due to glycation of complement protein 3. Secondary infections of peripheral regions occur with increasing regularity with progression of peripheral vascular disease. The increased oxidative stress that the disease causes can exacerbate secondary complications that are already present in the diabetic individual and lower their quality of life [22].

The risk factors associated with diabetes mellitus in America shown in the table below are quoted from Nathan, (1993) [31];

Complication	percentage risk of a diabetic developing the complication	ratio of incidence of complication compared to non-diabetics
visual impairment	14	-
blindness	16	20
renal failure	22	25
stroke	10	2-3
amputation	12	40
myocardial infarction (heart attack)	21	2-5
median survival after diagnosis of IDDM	36 years	
median age at death (IDDM)	49 years	

Note that IDDM is an abbreviation for insulin dependent diabetes mellitus.

### 1.2.1. Insulin therapy

Conventional insulin treatment consists of monitoring blood glucose concentrations on a daily basis and two daily insulin injections. The diabetes control and complications trial (DCCT) in America has shown that a strict insulin regime (i.e. at least 4 glucose checks which are used to self dose more than 3 insulin injections per day) does significantly reduce the risk of secondary complications, compared to conventional treatment. The achievement of the lower risk is by the very tight glycaemic control; i.e. the prevention of hyperglycaemia and thus excess glycation. Such a strict regime is not without its problems, apart from the inconvenience of so many checks and injections there is a 3 fold higher risk of hypoglycaemia and a tendency to gain weight [19, 38 and 39]. The first year of the strict regime after switching from conventional treatment has been reported to be problematic for diabetics with existing complications, e.g. retinopathy [40]. The strict insulin therapy reduces the rate of glycation considerably but not the progression of already glycated proteins to AGEs and thus secondary complications. In the patients studied it would appear that it takes one to two years before the build up of AGEs is limited by lowering the rate of glycation, such an occurrence would not, however, explain the initial exacerbation of the complications [39 and 40]. One explanation could be that the free glucose and glucose-protein adducts (intermediates to AGE formation) compete for reaction with lysine residues on other proteins, e.g. sites of glycation and AGE-cross-linking are the same. Therefore reducing the mean glucose concentration with the strict insulin therapy allows more cross-linking of glycated protein (the concentration of which may only fall slowly) to newly formed proteins. AGEs produced *in vivo* at a faster rate, in such a manner, would lead to exacerbation of diabetic secondary complications. If this hypothesis is true it implies that glycation



actually inhibits AGE formation and in the absence of free sugar post-Amadori or post-Heyns reactions should proceed at a faster rate; Eble *et al* (1983) reported just such an effect for polymerisation of RNase *in vitro* [185].

Continuous subcutaneous infusion is another method for administration of insulin. Lauritzen *et al* (1983) reported that this produced a near normal basal glucose concentration of 6mM, but that this was not enough to prevent progression of proliferative retinopathy in patients with simple retinopathy [41]. Lauritzen's study was carried out for up to a year which may have not been long enough to allow turnover of AGE-proteins and subsequent remission of the diseases. Alternatively the insulin therapy by this method was not strict enough to reduce the rate of AGE formation to below the rate of their removal.

The exacerbation of diabetic secondary complications, caused by a high rate of AGE formation upon maintenance of euglycaemia, could be prevented by administration of compounds that would inhibit post-Amadori or post-Heyns reactions. Such administration could cease after the first year of a strict insulin regime when the rate of glycation decreases to a level that the body can clear by protein turnover, as indicated by the studies described above [39 and 40].



### 1.3. Production of Advanced Glycation End-products (AGEs)

Figure 1.1. illustrates post-Amadori or post-Heyns reactions as a series of steps leading to production of AGEs, this is, however, an over-simplification of these reactions. There are probably many parallel multistep post-Amadori or post-Heyns reactions producing a wide variety of AGEs. The term AGE is itself a broad definition of a range of properties, e.g. fluorescence, chromophores, cross-linking and protein fragmentation. There is also evidence that these measures of AGEs may themselves be intermediates in the production of more AGEs; e.g. the production of brown pigmentation in food at the same time as a decrease in fluorescence [173]. Figure 1.3.1. illustrates the state of current knowledge of the AGEs produced from Amadori product.

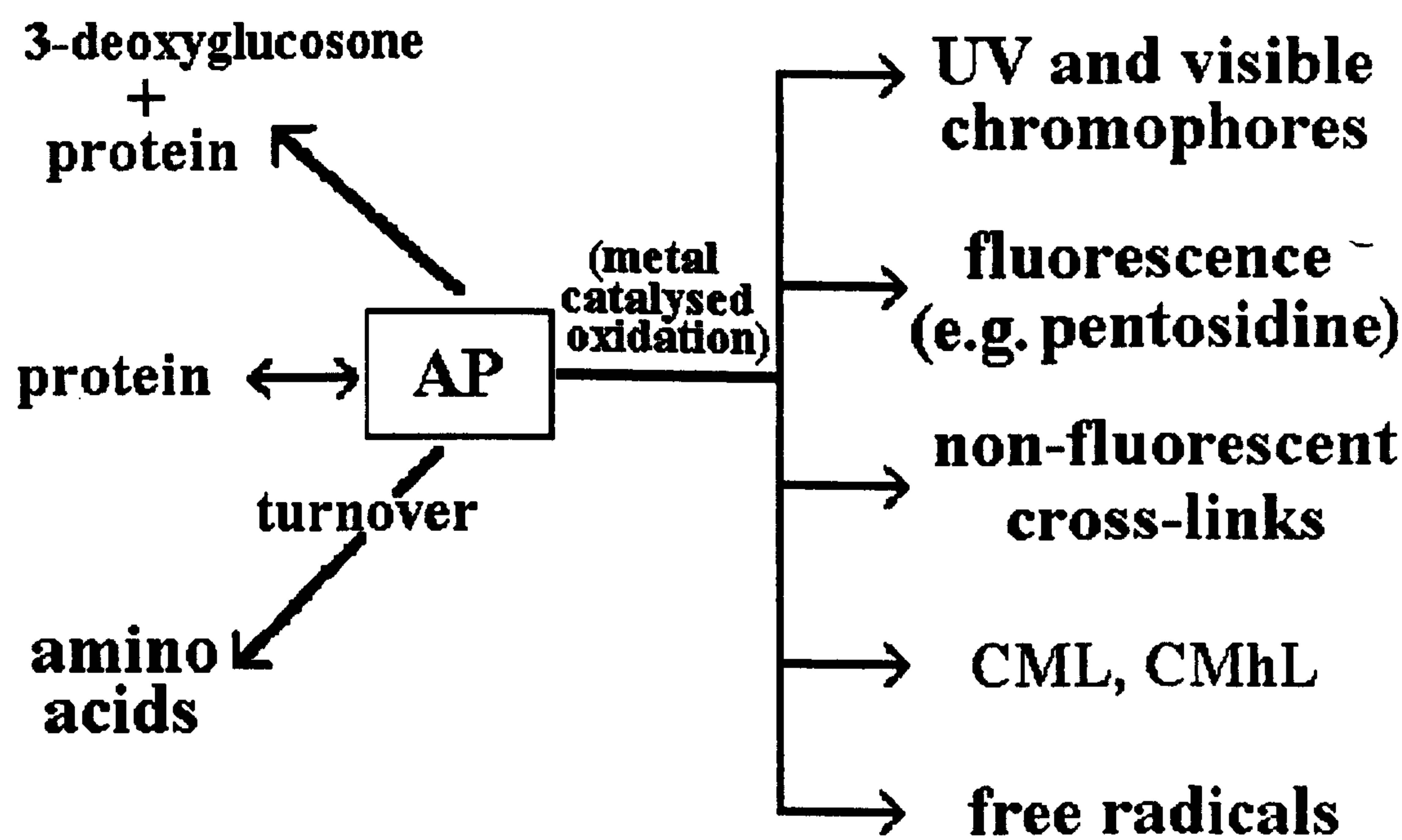


Fig. 1.3.1. Summary of post-Amadori reactions as we know them now. AP represents Amadori product, CML carboxymethyllysine and CMhL carboxymethylhydroxylysine.

The characterisation of post-Amadori or post-Heyns reactions would be useful for the rational design of compounds to inhibit formation of diabetic secondary complications. The most ideal solution for the prevention of diabetic secondary complication is to achieve stable euglycaemia such as exists in people without diabetes; however glycation of proteins that have a long half-life, or not even turned over at all, will eventually lead to the AGE formation that occurs in everyone, increasing as a function of age [23, 26, 29 and 30]. If the Maillard reaction *in vivo* was interrupted and halted it could delay the onset of some of the diseases associated with old age, this is of course assuming that diabetic secondary complications are signs of accelerated ageing. There is also a case for such therapeutic interruption to inhibit advancement of diabetic secondary complications during the first year of strict insulin therapy in patients who were previously conventionally treated [40, 39 and 41].

The next part of this section of the thesis is a brief description of the types of AGE illustrated in figure 1.3.1.

### 1.3.1. Fluorescence

The fluorescence produced as a result of glycation (AGE-fluorescence) has a very broad excitation and emission range, 300-400nm and 370-500nm respectively, probably because of the large number of compounds produced, which is further reflected by the variety of specific wavelengths quoted in the literature [10]. With the production of many similar fluorophores there is, inevitably, overlap of excitation and emission wavelengths and thus quenching. Apart from isolation of individual fluorophores by HPLC the literature suggests two sorts of approach to the problem. The first is to find which wavelength in the 300-400nm region that absorbs the most; excite at that wavelength and then scan at higher emission



wavelengths to find the maximum intensity; these two wavelengths are then used as the basis of the measurement. The second approach is to use the maximum fluorescence wavelengths of a known AGE biomarker, such as pentosidine, pyralline or furoyl-furanyl-imidazole (FFI), see figure 1.3.1.1. for their structures. The fluorescence wavelengths used in this thesis have tried to tackle the problems outlined above by using two sets of wavelengths. The first set corresponds to those of pentosidine as reported by Dyer *et al* (1991); i.e. excitation at 328nm and emission at 378nm, called pentosidine-linked fluorescence in this thesis [46]. The second set are those of the maximum of serum albumin after glucation or fructation as reported by Suarez *et al* (1989); i.e. excitation at 350nm and emission at 420nm, this I have termed albumin-specific fluorescence [14]. The problem with the first approach is that fluorescence does not necessarily derive directly from glycation alone, other fluorophores can occur under oxidative conditions (with or without the presence of saccharides). Products of lipid peroxidation, pyridium cross-links and tyrosine oxidation products are all fluorophores that will interfere with the assay of AGE-fluorescence [45].

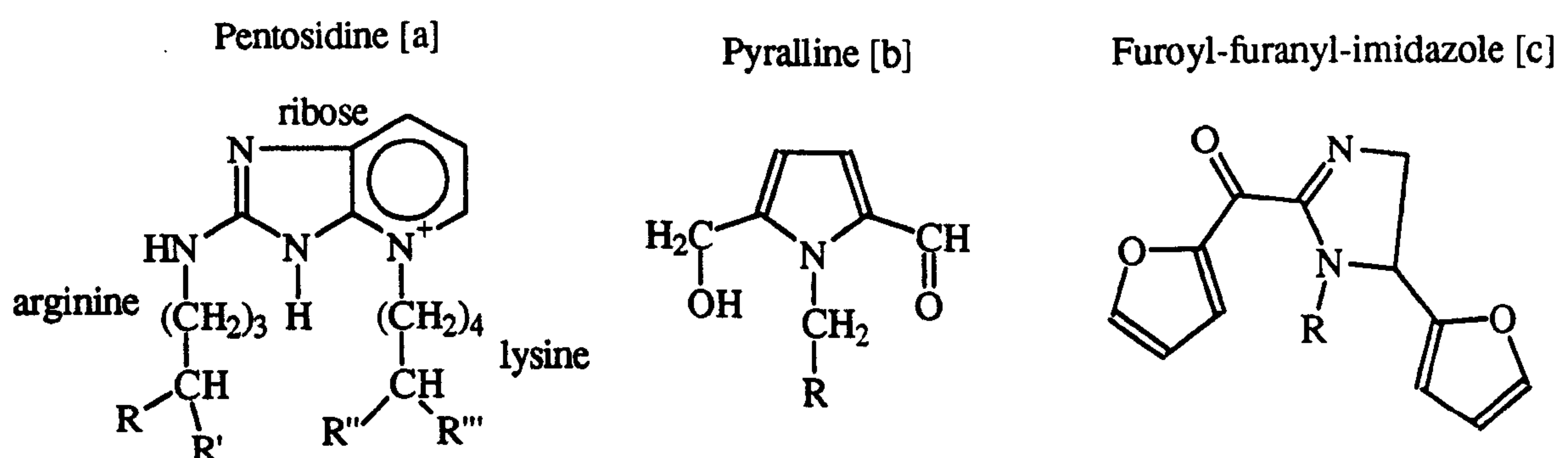


Fig. 1.3.1.1. Structures of some putative AGE biomarkers. The references in square brackets are; [a] = [42], b = [43] and c = [44]. R stands for the rest of the lysine residue and protein.

Pentosidine has been detected *in vivo* but *in vitro* forms less than 1% of AGE-cross-links detected and is thus only a minor product. The existence of pyralline *in vivo* has been cast in doubt by Smith *et al* (1993) and FFI has been reported to be an artefact of its own purification [46, 47 and 48].

### 1.3.2. Cross-linking

Pentosidine is not only a AGE-fluorophore, it is also a cross-link between lysine and arginine residues bridged by a ribose moiety, but note that pentosidine forms less than 1% of all AGE-cross-links formed by *in vitro* glycation [46 and 175]. AGE-cross-links can be subdivided into two distinct groups, those that are fluorescent and those that are not. Cross-linking can be measured as a function of increased resistance to degradation by chemicals and enzymes, which is the generally preferred method used to investigate proteins of high molecular weight such as collagen [187 and 178]. A second approach is to study the formation of oligomers of small proteins upon glycation, such as RNase, IgG, lysozyme, etc. [176, 185, 183 and 197]. The approach I have used in this thesis is the cross-linking of small proteins to form oligomers identified on denaturing discontinuous SDS-PAGE.

### 1.3.3. Protein fragmentation

Protein fragmentation caused by glycation and metal catalysed oxidation (MCO) is another example of AGE formation, MCO is discussed in greater detail in section 1.5.; but in relation to protein fragmentation:- Cheng *et al* (1991) reported that degradation of glycated protein in the presence of Cu(II) was not inhibited by scavengers of hydroxyl and super-oxide radicals; implying that, if free radicals were involved, their action was local to their site of formation [49]. Similarly Kawakishi, Okawa and Uchida (1990) reported degradation of BSA exposed to Cu(II) and fructose- $\beta$ -alanine or fructose-p-toluidine (which are Amadori



compounds found in food and can chelate copper); the enzyme catalase (CAT) inhibited the degradation but scavengers of free radicals, other than hydroxyl, did not [50].

#### 1.3.4. Carboxymethyllysine

Carboxymethyllysine and carboxymethylhydroxylysine (CML and CMhL) are degradation products of Amadori product. Cleavage between carbons 2 and 3 of the glucose residue of Amadori product results in CML and erythronic acid, see figure 1.3.4.1. [51].

Dunn *et al* (1989) reported that CML accumulated in lens protein as a function of age and the same group in (1991) reported the accelerated accumulation of CML in lens protein and skin collagen in diabetics compared to normal individuals [52 and 53]. CML and CMhL are carboxylic acids that have a pKa of around 3 (that is as long as the pKa is not modified by other local residues), which is very different to that of lysine (pKa = 10). If *in vivo* the lysine residue that had become glycated was involved in molecular recognition of the protein, such a change in charge could in theory inhibit this recognition and alter the properties of the protein.

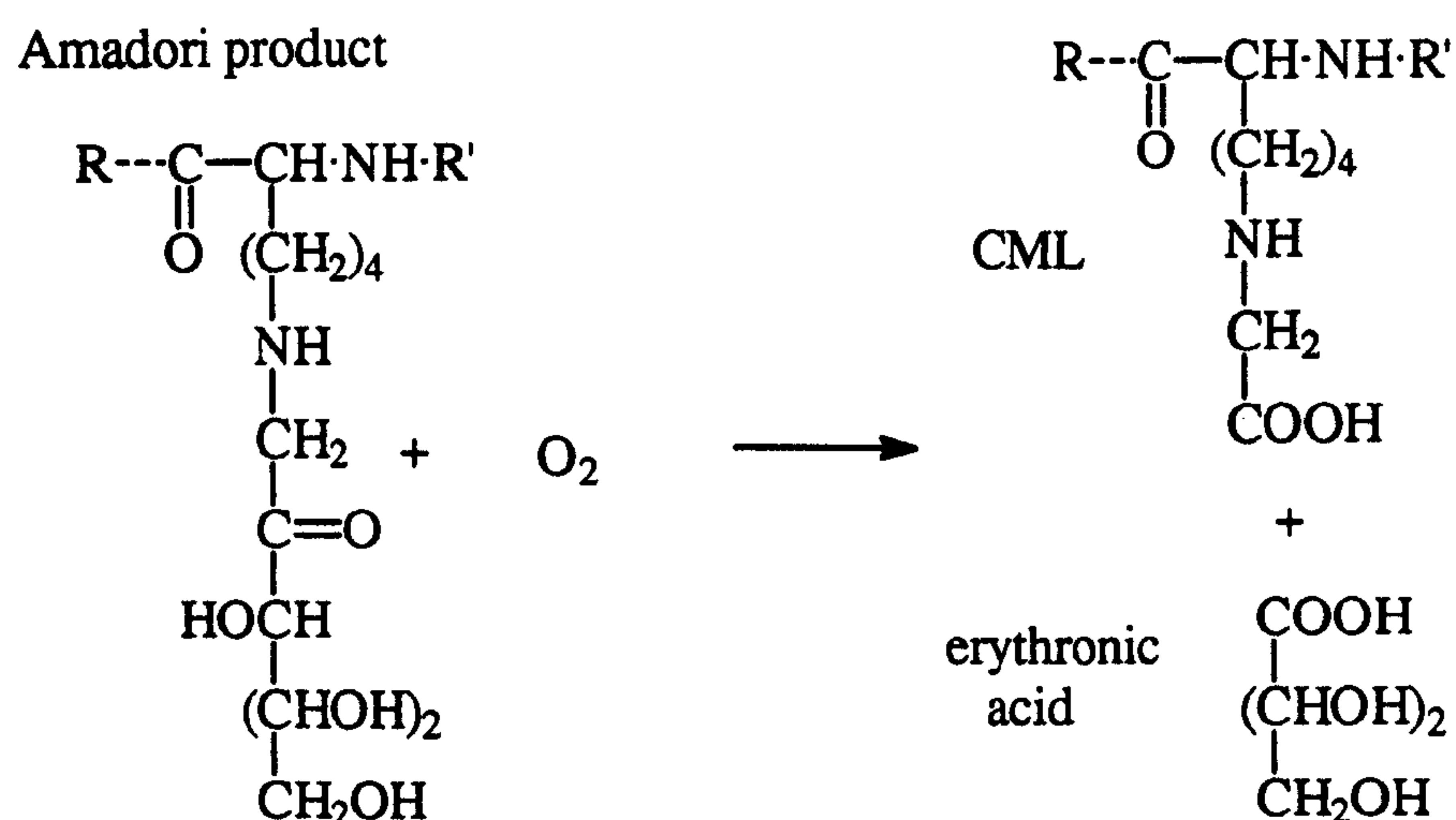


Fig. 1.3.4.1. Formation of CML from Amadori product, reproduced from Fig. 3 of Ahmed, Thorpe and Baynes (1986) [51].

### 1.3.5. AGE-chromophores

The browning reaction is what food scientists sometimes use to refer to the Maillard reaction that occurs during the cooking of foods, to produce brown pigmentation. The reaction of reducing sugars and the primary amino groups of free amino acids in foods is an important source of the 'brown' chromophores; proteins will also absorb in the visible region of the spectrum when exposed to reducing sugar [13]. *In vivo* chromophores that absorb in the visible region are thought to occur on proteins although the melanioidins frequently quoted may also be products of lipid peroxidation. The absorbance of AGE-fluorophores can also be measured in the UV region [10].

### 1.3.6. Free radicals

Mullarkey *et al* (1990) reported that free radicals were produced from glycated protein at a rate some fifty times that of non-glycated protein [74]. In contrast advanced glycation end-products have been reported to exhibit free radical scavenging properties [75]. Free radicals generated *in vivo* can result in oxidative stress, such stress probably exacerbates existing disease processes (e.g. diabetic secondary complications), this is particularly important in people of older age because the levels of super-oxide dismutase (SOD) and CAT in the blood decrease with age [70].

## 1.4. Assays for glycation and AGEs

This section of the thesis is a description of the various assays used to measure glycation and the subsequent AGE formation that occurs both *in vivo* and *in vitro*. The measurement of *in vivo* glycation is important to monitor the degree of glycaemic control a diabetic has had over the life-span of the protein assayed; this is important for calculating the dosage of insulin to be administered. *In vitro*, the assays are used as research tools for the study of the Maillard reaction. Although not all the assays described below have been used in this thesis, this is a useful point at which to include them for the reader's interest.

**Glycated haemoglobin** is used to monitor the glycaemic control a diabetic has had over the past few months. Proteins that have been glycated have a lower pKa than their native counterparts (due to the modification of the lysine residue), thus separation of glycated Hb is achieved using its altered isoelectric point. N-terminal glycation produces a species of Hb called HbA<sub>1</sub> that encompasses four subspecies designated HbA<sub>1a1</sub>, HbA<sub>1a2</sub>, HbA<sub>1b</sub> and HbA<sub>1c</sub>. Glucose produces HbA<sub>1c</sub> and this fraction contributes about 4% to the total haemoglobin in normal patients but in uncontrolled diabetes up to 20% has been observed [148]. Different methods used to assess glycaemic control use different Hb subfractions; the methods range from measurement of total glycated haemoglobin (GHb, all subfractions), through to HbA<sub>1c</sub> on its own. More thorough reviews of the measurement of glycated haemoglobin can be found in Mortensen (1982), Standing (1992) and Kilpatrick (1994) [54, 148 and 55].

**The serum fructosamine assay** measures ketones under alkali conditions and is aimed at measuring Amadori product of HSA; figure 1.4.1. illustrates the mechanism of the fructosamine assay as proposed by Baker *et al*, (1994) [58]. The



assay relies on the production of an eneaminol from Amadori product under alkali conditions, this in turn reduces the nitroblue tetrazolium to form a blue black colour that can be quantified spectrophotometrically. The method is difficult to standardise because the colour varies with the protein, dye concentration, pH, temperature, interfering compounds (aldoses; e.g. glucose and Schiff's base) and assay time. Koskinen *et al* (1992) and Das *et al* (1992) have reported problems with the assay in clinical laboratories that are due to the non-specificity of the proteins used (all serum proteins) and have reported that only about half of a positive result is due to glycated protein [56 and 57].



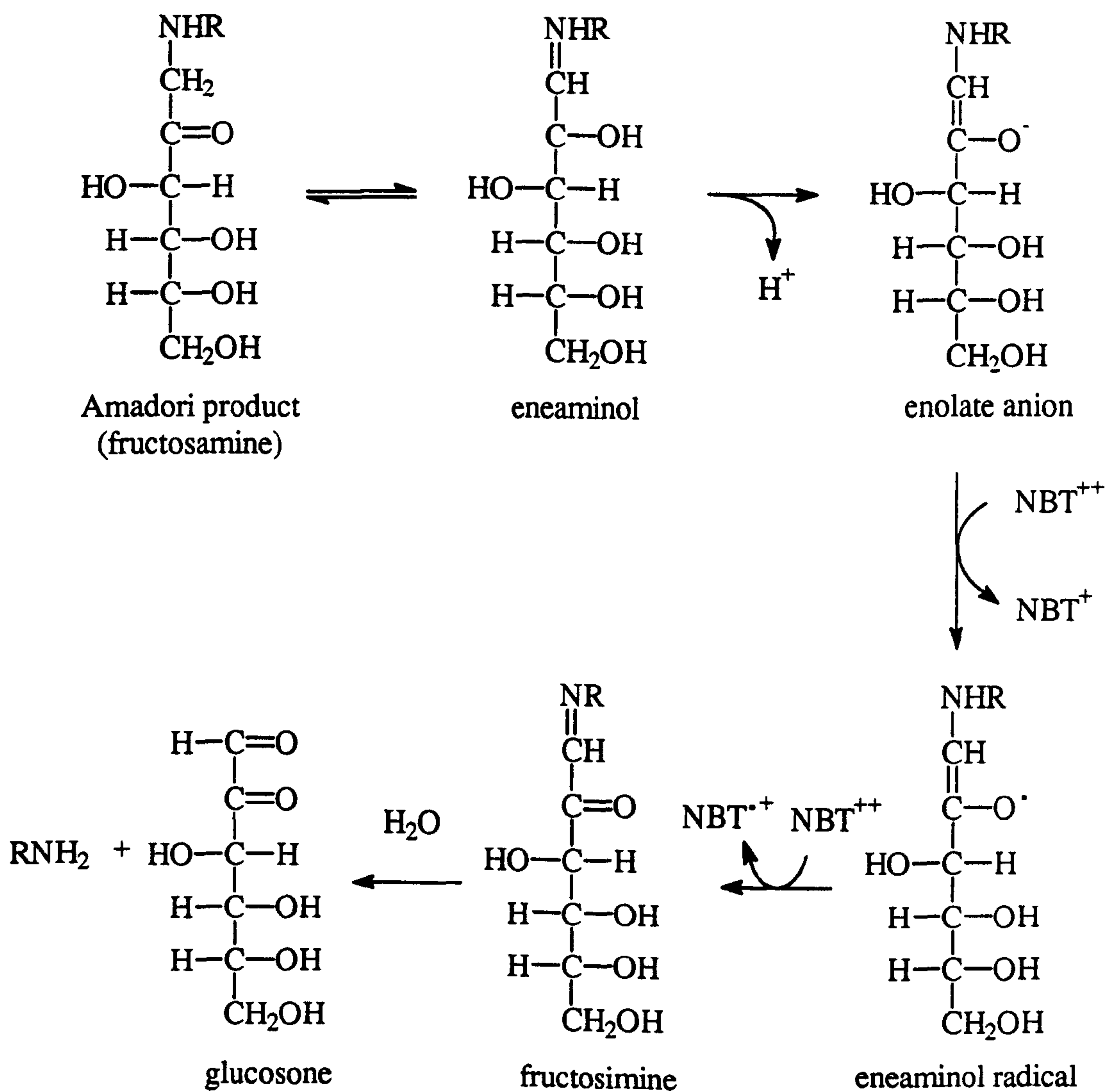


Fig. 1.4.1. The postulated reaction mechanism for formation of reduced NBT from Amadori product. Reproduced from Fig. 6 of Baker *et al* (1994) [58].

**The periodate assay** relies on the release of formaldehyde upon cleavage of adjacent carbons or C-N bonds carrying hydroxyl or carbonyl groups upon reaction with sodium periodate. In practice this means that the structures known to give a positive result include free sugars, Schiff's base, Amadori product and chain terminal reducing sugars of glycoproteins; thus to measure Amadori product the other compounds must be removed. There are two possible isomers of Heyns product of which only the minor form will yield formaldehyde upon periodate oxidation. AGEs or their precursors may also yield formaldehyde upon periodate oxidation, this is however difficult to quantify without knowledge of the structures of these compounds. A description of the assay protocol can be found in section 2.7.

**The thiobarbituric acid (TBA) assay** measures hydroxymethylfurfural (HMF) released by acid hydrolysis of sugars with 6 or more carbon atoms. It measures free sugar, Schiff's base, Amadori product, and the carbohydrate residues of glycoproteins if they are acid labile. Thus to measure glycation, free sugars and glycoproteins have to be removed prior to the assay. A similar assay is used to measure lipid peroxidation and the reactive substances are termed thiobarbituric acid reactive substances (TBARS).

**Phenylboronate chromatography** relies on the complexation of the column matrix with coplanar cis-hydroxyl groups such as those on Amadori product. A solution of glycated protein is applied to the column, non-glycated protein is eluted off and glycated protein remains on the column. Comparison of the protein concentration in the solution applied to the column with that of the eluted solution allows calculation of the amount of protein that has become glycated. Problems with the method include; the non-detection of products of glycation that do not have

coplanar cis-hydroxyl groups, interference by glycoproteins and non-quantification of glycation at more than one site on a protein.

Shima *et al* (1988) coupled phenylboronate chromatography with HPLC to assay for glycated human serum albumin [61]. Increased glycation of apolipoprotein B in diabetics (types 1 and 2) was reported by Panteghini *et al* (1994) using phenylboronate chromatography coupled to immunonephelometry [62].

**Sodium borohydride** reduces Schiff's base and Amadori product to deoxyhexitols that are acid stable; the glycated amino acids are then identified by acid hydrolysis of the protein followed by amino acid analysis. This method can be complimented with mass spectrometry or enhanced by the use of tritiated borohydride.

The **furosine assay** was developed by Schleicher and Weiland (1981) to specifically detect Amadori product [63]. The method relies on strong acid hydrolysis of Amadori product (6M HCl, 95°C for 18 hours) to form furosine which can be quantified on reverse phase HPLC.

**The development of AGE-chromophores and fluorescence** are characteristics of AGEs that are almost universally used throughout the literature. A problem with fluorescence is the lack of characterisation of fluorescent AGEs and the enhancement or quenching of their fluorescence by other AGEs, thus forming very broad fluorescence spectra.

**Reduced activity of enzymes upon glycation** is potentially a very sensitive technique; Dolhoffer (1982) and Ganea (1988) reported the loss of activity in a variety of enzymes as a result of glycation using physiological concentrations of sugars [64 and 65].



**AGE-cross-links** can be detected either by the increase in molecular weight using methods such as SDS-PAGE with denaturation of disulphide bridges (discussed in more detail in chapter 5.), or by resistance to degradation by chemicals and enzymes, which is a useful way of measuring glycation in large proteins such as collagen [179].

**Molecular recognition of AGEs by antibodies** is another potentially powerful technique but suffers from the lack of characterisation of AGEs and is only as specific as the method used to select the antigen or antibody. Cohen *et al* (1989) reported the use of monoclonal antibodies raised against *in vivo* glycated human serum albumin, isolated by phenylboronate affinity chromatography [66]. In (1993) the same group reported glycated apolipoprotein B concentrations in non-diabetics and diabetics using the same method [67].

Excellent reviews of the methods described above include Furth (1988) and Schleicher and Wieland (1989) [59 and 60].



## 1.5. Glycooxidation

The term glycooxidation was coined to emphasise the link between AGE formation and oxidation, specifically metal catalysed oxidation. *In vitro* AGE-cross-linking of collagen, formation of CML, fluorescence and protein aggregation requires oxidative conditions. Glycation under nitrogen (exclusion of oxygen); inclusion of the metal chelators diethylenetriaminepentaacetic acid (DTPA) or desferrioxamine; the reducing agents dithiothreitol (DTT) or penicillamine; the free radical scavengers' thiourea, mannitol (hydrogen peroxide, hydroxyl and super-oxide radicals) and the enzyme catalase (CAT) all inhibit AGE formation with a corresponding build up of Amadori product [134, 179, 177 and 72]. Glycooxidation requires free metal because oxygen-centred free radicals cannot directly react with biological molecules due to spin restriction, thus it requires the intervention of free metal ( $\text{Cu}^{\text{II}}$  or  $\text{Fe}^{\text{III}}$ ) to relieve the restriction [68]. Free copper<sup>II</sup> can, at high concentrations (200 $\mu\text{M}$ ), induce BSA to polymerise; however *in vivo*, there is only about 1.5 $\mu\text{M}$  copper<sup>II</sup> that can take part in oxidative reactions in the serum and this is mostly bound to albumin; the majority of copper in circulation is stored bound in caeruloplasmin (about 14 $\mu\text{M}$  in normal individuals). Total serum copper increases as a function of age while concentrations of SOD, glutathione peroxidase and CAT are stable until old age whereupon they decrease. Thus, an increase in oxidative stress could be a major factor in the pathogenesis of diabetic secondary complications in non-insulin dependent diabetes mellitus (NIDDM). [69 and 70]. The phosphate buffer used in this thesis contains free copper and iron at a concentration that varies according to the stock used but both have a maximum concentration of between 2 and 5 $\mu\text{M}$  [207].

Le Guen *et al* (1992) reported that the inclusion of SOD during *in vitro* glycation accelerated the development of AGE-fluorescence and concluded that hydrogen peroxide, free metal and hydroxyl radicals were all involved in AGE formation [72]. The generation of free radicals by Amadori product has also been reported to be a mechanism for the further oxidative damage of protein and protein with lipid [73 and 74]. Conversely melanoidins, the term used for brown pigmented AGEs and products of lipid peroxidation, are reported to be scavengers of free radicals [173 and 75].

Metal catalysed oxidation and free radicals have been linked to the pathogenesis of many diseases associated with ageing; e.g. Alzheimers disease, cataractogenesis, pulmonary emphysema and exacerbation of tissue injury after stagnant hypoxia (caused by return of blood flow after, for example, stroke or heart attack) [76, 77 and 78]. Inflammatory diseases such as rheumatoid arthritis have been attributed to oxidative stress caused by the presence of oxidatively modified IgG. Neutrophils react to the modified IgG by releasing super-oxide to destroy it, but the super-oxide also oxidises previously unaltered IgG perpetuating the disease [79]. A similar mechanism has been suggested by the same group to be involved in the pathogenesis of atherosclerosis [78]. The accelerated ageing seen in diabetes could be linked to the co-ordination of free metal to the sugar moiety of glycated protein; the complex formation is likely since saccharides will strongly complex first row transition metals [80]. There are other free radicals produced *in vivo* that could exacerbate existing disease processes. Nitric oxide is now known to be the endothelial relaxing factor. Nitric oxide has been reported to cause release of iron from ferritin and in places of low super-oxide concentration, such as atherosclerotic plaques, acts as an oxidant [81 and 82]. Nitric oxide will also react with super-oxide (without the influence of free metal) to produce another free

radical called peroxynitrite that has been reported to be about 2000 times as reactive as hydroxyl radical in the initiation of lipid peroxidation and DNA scission [82]. Free radicals derived *in vivo* from sources other than free metal are receiving increased attention among researchers; iron-containing proteins have been reported to produce free radicals which could contribute to disease processes [71].



## 1.6. Glycation by sugars other than glucose

Glucose is present in circulation, *in vivo*, at a concentration much higher than any other sugar. Glucose also exhibits the lowest percentage open chain form (0.002% aldehyde at 20°C in aqueous solution) of any of the reducing sugars surveyed by Angyal (1984) [15]. Bunn and Higgins (1981) surveyed the reactivity of a range of monosaccharides with haemoglobin and found that glucose forms an adduct with the protein at a very low rate compared to other reducing sugars. The low percentage of open chain form was postulated to be the reason for the low reactivity of glucose with haemoglobin; hence glucose is the universal primary metabolic fuel rather than any of its stereoisomers [83]. The Maillard reaction with respect to glucose can be termed glucation to distinguish it from other sugars and is illustrated in figure 1.6.1.

The food industry has long been concerned with sugars other than glucose because of their abundance in food and the fact that they form AGEs more rapidly than glucose. Burton and McWeeny 1963 reported that mannose, galactose and, in general, all ketoses formed chromophores with glycine at a faster rate than glucose [84].

This thesis is concerned with *in vivo* glycation where, with the exception of glucose, free reducing sugars are scarce. The human gut will absorb three monosaccharides (glucose, galactose and fructose) all of which are detected in circulation. Galactose is absorbed in the small intestine by active transport and its concentration prior to removal by the liver is dependent on the concentration in the ingested food or drink; in general circulation its concentration rarely rises above 1mM and has a basal concentration of around 0.1mM [85 and 207]. A major dietary source of galactose is in the form of lactose in milk. Deficiency of the



enzymes of galactose metabolism (three recessive disorders) causes galactosaemia, the symptoms of which are mental retardation, sugar cataracts and even death if untreated. The formation of cataracts due to galactosaemia is assumed to be analogous to that of diabetic cataract [85]. Fructose is available *in vivo* from two sources, dietary intake and the polyol pathway.



### 1.6.1. Dietary fructose and its metabolism

Fructose is naturally abundant in the western diet in the form of a monosacharide (honey, apples, pears) and as a disacharide (sucrose) [89]. Fructose as a monosacharide is sweeter than sucrose, thus the use of high fructose corn syrups (HFCS) as sweeteners, has become more prevalent in the food industry; up to 90% of the carbohydrate in HFCS is fructose. HFCS are used primarily in the manufacture of soft drinks and convenience foods; their use has increased dramatically over the past 25 years both as a function of their substitution of sucrose and with the increased consumption of these goods [89 and 90].

The average daily consumption of fructose varies in the literature, the majority of information originates from figures of food consumption issued by the US department of agriculture. Swanson *et al* (1992) reported 46g per day as an average for the population of America, this represents 10% of total daily calorific intake [91]. Park and Yetley (1993) (using the same data) reported 15g per day in infants, 54g per day in 15 to 18 year old males with an average for the whole US population of 37g per day which represents 7-9% of total calorific intake [90]. Previous reports using the same source of information have also put the estimate of daily intake at 70g or between 50 and 100g mostly in the form of sucrose [92, 93 and 96]. Rumessen (1992) reported that when sucrose consumption is fully taken into account the highest consumers (15 to 18 year old males) consume an average of 150g daily which the article claims to be 4% of the total daily calorific intake [89].

If 50g (as a 10% solution) of fructose is ingested without the presence of another monosaccharide, 37.5% of the population will malabsorb fructose, producing bloating, flatulence and diarrhoea; this figure rises to 71% of the population if the



50g is ingested as a 20% solution. Fructose absorption is stimulated by simultaneous ingestion of glucose in a dose dependent manner, malabsorption only occurs if fructose is in excess of glucose [89]. Fructose is actively transported by a sodium-dependent stereospecific carrier called GLUT5 at a rate 60-90% that of glucose; the rate is partly dependent on the carrier concentration which in turn is dependent on the historical fructose content of the diet [94, 89 and 95]. The concentration of fructose in the hepatic portal vein is largely dependent on the oral load and up to 2.5mM has been reported, while intravenous infusion of 1.5g per kg per hour produced 7mM [96].

Figure 1.6.1.1. shows a diagram of the metabolism of fructose as adapted from Shafrir (1985) and Gitzelman *et al* (1989) [standard texts of glycolysis, 93 and 96]. Fructose enters the liver by insulin independent active transport, here the enzyme fructokinase adds a phosphate group to carbon-1. Fructose-1-phosphate is a substrate of aldolase which converts it to glyceraldehyde and dihydroxyacetonephosphate which then enter the glycolytic pathway.

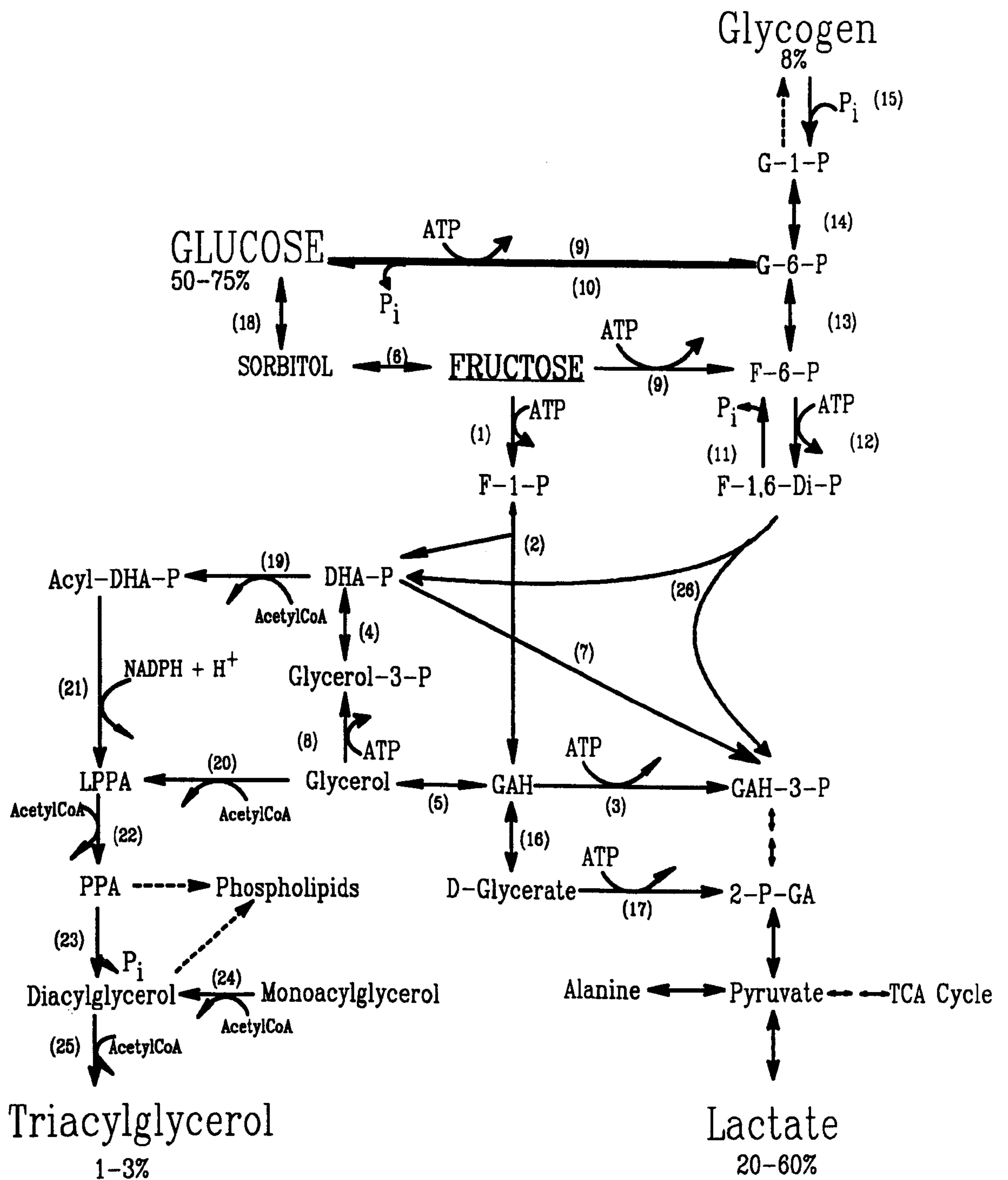


Fig. 1.6.1.1. Metabolism of fructose, the proportion of the products is represented by the percentage figures. Adapted from standard texts of glycolysis and refs. 93 and 96.

Key to figure 1.6.1.1.

Abbreviations; DHA = dihydroxyacetone; GAH = glyceraldehyde; F = fructose; G = glucose; GA = glycerate; LPPA = lysophosphatic acid; PPA = phosphatic acid:

Enzymes; 1) fructokinase; 2) aldolase B; 3) triokinase; 4) glycerol-3-P dehydrogenase; 5) alcohol dehydrogenase and aldose reductase; 6) sorbitol dehydrogenase; 7) triose phosphate isomerase; 8) glycerol kinase; 9) hexokinase and glucokinase; 10) glucose-6-phosphatase; 11) fructose-1,6-bisphosphatase; 12) phosphofructokinase; 13) phosphohexose isomerase; 14) phosphoglucomutase; 15) glycogen phosphorlyase; 16) aldehyde dehydrogenase; 17) glycerate kinase; 18) aldose reductase; 19) dihydroxyacetone phosphate acyltransferase; 20) glycerol-3-P acyltransferase; 21) acyl-dihydroxyacetone phosphate reductase; 22) 1-acylglycerol-3-P acyltransferase; 23) phosphatidic acid phosphatase; 24) 2-monoacylglycerol acyltransferase; 25) diacylglycerol transferase; 26) aldolase A & C.



The high reactivity of fructokinase has been implicated in the production of hyperuricaemia and lactic acidosis that is observed with high doses (>1g per kg per hour) of intravenously administered fructose [96]. The lactic acidosis occurs because fructose metabolism bypasses phosphofructokinase which is the first regulatory enzyme of glycolysis and thus leads to a build up of lactate, see figure 1.6.1.1. Hyperuricaemia occurs because the phosphorylation of fructose, by fructokinase, causes a build up of ADP and depletion of inorganic phosphate. This change triggers adenylate kinase to convert 2ADP to ATP and AMP; a build up of AMP is prevented by its catabolism, by AMP deaminase, to IMP which in turn is dephosphorylated to inosine (cytoplasmic-5-nucleotidase). Inosine is catabolised in 2 steps, by nucleoside phosphorylase and xanthine oxidase, to produce uric acid and 2NADH [93 and 96].

Figure 1.6.1.1. shows the end products of fructose catabolism as ranges of percentages, the proportions of the final products formed are dependent on the concentration of fructose ingested [96]. High oral fructose loads have been reported to lead to increase the intracellular concentration of hexose phosphates which then inhibits glucose uptake in the liver [93]. This could have profound implications on the degree of hyperglycemia experienced by diabetics. The increased concentration of serum triacylglycerol caused by diets with normal western intake of fructose has been linked to hyperlipidaemia, atherosclerosis and coronary artery disease but is also affected by age, sex (hormone status), average serum glucose concentration, insulin concentration and resistance to it [93, 97 and 98]. Hyperinsulinaemic men are more susceptible to hyperlipidaemia when consuming average amounts of dietary fructose than the same men on low fructose diets or normal controls [92].

### 1.6.2. Concentration of fructose in general circulation

The high rate of uptake of fructose by the liver ensures that its concentration in the blood of general circulation is very low with a basal range of 0.03 to 0.1mM; the range represents differences in the methods used to detect fructose [99]. Marks and Flatt (1989) reported that serum fructose never exceeds 1mM upon an oral load while Gitzelmann reported 0.35mM [85 and 96]. Haber *et al* (1977) studied serum fructose after eating apples, apple purée or drinking apple juice; after an overnight fast a maximum of 0.44mM was observed 1 hour after the meal. The apple meals contained 60g of available carbohydrate of which 10% was fructose (as a monosaccharide or as sucrose); the largest rise was observed with consumption of 444ml of juice [100]. MacDonald *et al* (1978) reported a maximum fructose concentration, in the blood, of 0.53mM (SE  $\pm$  0.03mM) 1 hour after ingestion of 1g per kg (25% solution), after an overnight fast; a rise in serum fructose was approximately half when fructose was substituted with sucrose [101]. A maximum serum fructose concentration of 0.41mM was reported after ingestion of 75g of fructose (as 25% solution) by fasted normal healthy individuals [102].

Some pathologies adversely affect serum fructose concentrations upon an oral load. MacDonald and Turner (1971) studied atherosclerotic men and obtained maximum serum fructose concentration of ~0.8mM when, after an overnight fast, they ingested 2g per kg of sucrose (as a 100% solution), while age matched normal controls exhibited a similar response as normal young men, i.e. a maximum of ~0.3mM. Notably in this study the serum glucose concentrations in the atherosclerotic men were no different to their age matched controls yet in the former group the fructose concentration rose higher and remained elevated for longer [103]. Higher serum fructose concentrations were also observed, after administration of 100g of sucrose, in elderly type 2 diabetics compared with



normal healthy young controls [104]. Bantle *et al* (1986) studied serum fructose concentrations of type 1 and 2 diabetics after starch (control), sucrose and fructose diets; the percentage of total calorific intake of these diets contributed by fructose were <5%, 11.5% and 21% respectively. The starch diet gave a maximum serum fructose concentration of 0.1mM in both types of diabetics; the sucrose diet gave 0.2mM in type 1 and 0.3mM in type 2 diabetics; the fructose diet produced a maximum of 0.3mM in both types of diabetic although one individual had a concentration of 0.9mM [105]. The one individual that exhibited a fructose concentration of 0.9mM, in their blood after a fructose meal, could have had undiagnosed liver cirrhosis; liver disease would result in such a loss of its function. I have made this suggestion on the basis that glucose intolerance and liver cirrhosis are diseases that often occur together [106]. Avgerinos *et al* (1992) reported maximum serum fructose concentrations of 0.3mM in people with normal liver function upon ingestion of a meal high in sucrose, while cirrhotics with good liver function had a maximum of 0.8mM and those with poor liver function 2mM upon ingestion of the same meal [107].

The studies described above indicate that the concentration of fructose in the blood of general circulation is, with a normal balanced diet, never very high. The concentration does however increase after ingestion of foods that contain fructose. This concentration may be high enough to cause a rate of fructation beyond that which the body is able to destroy by turnover of proteins. Fructation from dietary fructose is particularly relevant to diabetics because of its use in foods recommended for their consumption. Diabetics produce AGEs from glucation that contribute to the formation and progression of diabetic long term complications, fructation can only add to this load and exacerbate the complications.



### 1.6.3. The Polyol Pathway

The polyol pathway seems to occur in tissues that do not need insulin for the uptake of glucose. It is of great interest that such tissues also exhibit the long term complications of diabetes, i.e. lens, retina, cornea, nervous system and kidney. The pathway consists of the two enzymes aldose reductase and sorbitol dehydrogenase and it converts glucose to fructose via sorbitol, see figure 1.6.3.1.

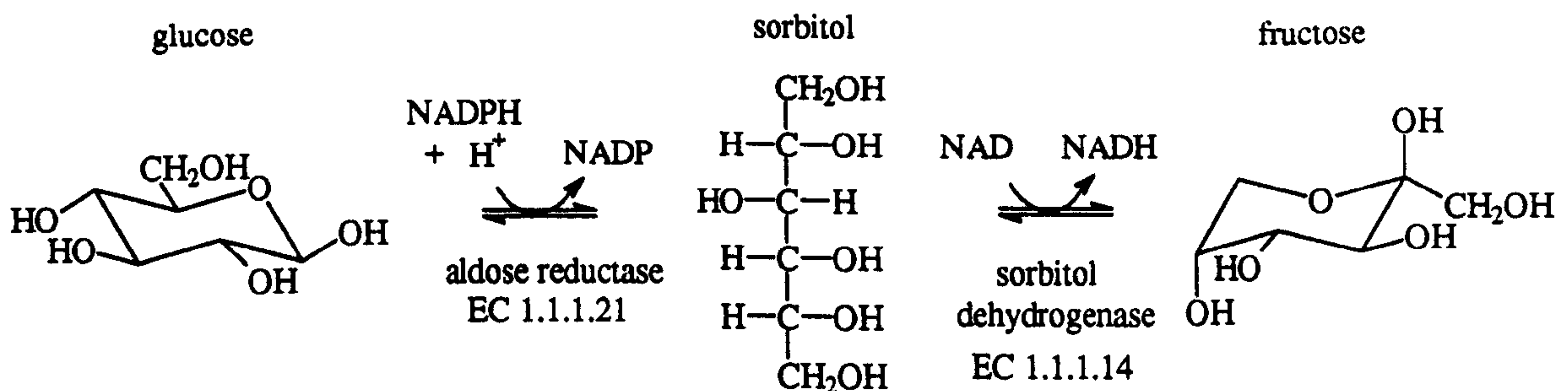


Fig. 1.6.3.1. The polyol pathway.

Aldose reductase is the most extensively studied enzyme of this pathway because it can be inhibited by a wide variety of compounds (termed 'aldose reductase inhibitors') of which sorbinil is the most widely known. Prevention of accumulation of sorbitol by such inhibitors has been hailed as a possible therapeutic measure for the prevention of diabetic secondary complications. This conclusion relies on the osmotic effect of sorbitol which, as a sugar alcohol, poorly penetrates cell membranes and thus accumulates and exerts intracellular osmotic pressure. The resultant over-hydration could disrupt a collagen matrix within a tissue such as the cornea and produce the effects seen in the secondary complications of diabetes. Aldose reductase has a low affinity for glucose, thus only binds glucose during hyperglycaemia. The accumulation of sorbitol during hyperglycaemia is an alternative theory to glycation and AGE formation for the

development of diabetic secondary complications and is extensively reviewed by Cohen (1987) [108].

Cohen (1987) in her review reported that sorbinil prevented progression of diabetic retinopathy in NIDDM patients, improved corneal re-epithelialization of an IDDM woman with keratopathy and improved neural and glomerular Na/K-ATPase activity in diabetics [108]. Aldose reductase inhibitors do not however prevent glucation or glycation by catabolites of glucose; Lorenzi *et al* (1987) reported a three fold accumulation of glucose-6-phosphate (from glucose via hexokinase) in cultures of human endothelial cells, from human umbilical veins, when exposed to 20mM glucose and 0.1mM sorbinil [109].

Suarez *et al* (1988) reported inhibition of skin collagen AGE-fluorescence in diabetic rats treated with sorbinil; i.e. some of the *in vivo* fluorescence was derived from fructose. Additionally the AGE-fluorescence spectrum of the collagenase solubilised skin was indistinguishable from that of fructated BSA [110]. The same group in (1989) reviewed the fructose concentrations within various tissues with active polyol metabolism. In the 15 papers cited, the fructose concentration within the tissues of normal individuals was not less than 0.05mM and was much higher in diabetics, not less than 1.24mM. In the lens of a human diabetic, fructose concentrations up to 12mM have been cited [111]. The activities and concentrations of the enzymes of the polyol pathway differ between tissues and species, thus the ratio of sorbitol to fructose also varies, for example in the human lens, fructose is the major metabolite [108].



#### 1.6.4. *In vitro* fructation

Ketoses in general have a lower reactivity than aldoses (approximately fifty fold lower), but the rate of glycation is also dependent on the relative concentration of the acyclic, reactive form of the sugar. Therefore fructose, which is 0.7% acyclic at 20°C in aqueous solution, has a potential reactivity with primary amines some 7.5 times faster than glucose [15 and 133]. This figure corresponds well to the observed rate of reaction of fructose with haemoglobin reported by Bunn and Higgins (1981) (an *in vitro* study that used reduction by [H<sup>3</sup>]-sodium cyanoborohydride to trap Schiff's base) [83]. The figure for the percentage of acyclic fructose has been reported by Yaylayan *et al* (1994) to have been underestimated by Angyal (1984) (0.7%), the proportion of the high energy open chain form of monosaccharides depends on the temperature and pH of the solution that contains it [86 and 15]. I have termed the Maillard reaction with respect to fructose, fructation, to distinguish it from glucation; a diagram of the known chemistry of fructation is illustrated in figure 1.6.4.1.

The table in figure 1.6.4.2. is a summary of the results of a number of publications investigating fructation. It would be presumptuous to compare the actual figures presented in these articles with each other because of the different experimental conditions used. The literature also tends to confuse the picture of fructation with the use of assays for glycation that underestimate fructation [209]. An example of this is the furosine assay adopted by Sakai *et al* (1990), the assay only detects Amadori product, thus any positive result produced from fructation is probably due to rearrangement of Heyns to Amadori products [198]. The conclusions that can be drawn by comparison of the literature include:



The higher rate of Schiff's base formation from fructation of haemoglobin compared to that of glucation reported by Bunn and Higgins (1981) supports the evidence for fructose having a higher percentage of reactive acyclic form than glucose [83]. Lapolla *et al* (1994) measure glycation as a function of increases in the molecular weight of a protein, i.e. a sugar-protein adduct has a higher molecular weight than native protein. The results of this article indicate that incorporation of fructose into RNase is at least three times faster than glucose [115]. A higher rate of reactivity for fructation compared to glucation is also supported by the higher rate of AGE formation from fructose cited in the literature.

The above paragraph logically concludes that because the concentration of the reactive form of fructose is higher than that of glucose, the rate of AGE formation would be higher upon glycation with the former. There are, however, two articles that confuse this neat scenario. Suarez *et al* (1989) reported that glucose and fructose blocked the primary amino groups of BSA at the same rates, yet fructation yielded ten times more fluorescence [14]. A later paper by the same group suggested that a possible explanation for this phenomenon could be; free fructose covalently reacts with the amino group of Heyns product such that two fructose residues are bound to a single lysine residue; the two fructose residues may in this way form a pyrrole by aldol condensation [87]. The second paper was by McPherson *et al* (1988) who reported that [U- $^{14}\text{C}$ ]-glucose was incorporated at the same rate as [U- $^{14}\text{C}$ ]-fructose into RNase, yet AGE formation on RNase was faster upon fructation than glucation. These results are not logically consistent, similarly the same paper reported that glucose was incorporated into HSA some 8 times faster than fructose, which is inconsistent with rates of AGE formation, for the two sugars, presented throughout the literature. A duplicate experiment carried out by myself is presented later in this thesis [88].



**Fig. 1.6.4.2. *In vitro* studies of fructation;**

sugar concentration	protein studied	results			Ref.
0.002 to 0.05M	Hb	rates of reduction with borohydride			[83]
		glucose	0.0006	per mM per hour	
		fructose	0.0045	per mM per hour	
0.2M		polymerisation after 28 days glycation			[112]
		glucose	fructose		
	BSA	>1%	~14%		
	RNase	>7%	~18%		
	lysozyme	>7%	~40%		
0.5M	RNase		fluorescence	polymerisation	[88]
		at 15 days	per mg	(% oligomer)	
		glucose	5	3	
		fructose	15	17	
0.5M	BSA	after 50 days glycation comparison of glucation with fructation showed; the same number of blocked amino groups; fructation exhibits more absorbance and 10 times more fluorescence than glucation.			[14]
0 to 0.1M	BSA	fluorescence (units/mg)			[113]
		sample	7 days	14 days glycation	
		control	0.128	0.358	
		glucose	0.377	1.1	
		fructose	0.82	2.4	
		sorbitol	0.13	0.37	



Fig. 1.6.4.2. continued.

0.2M	collagen	fluorescence (units per mg)				[199]
		days of incubation				
		sample	0	7	14	
		control	0.84	1.14	1.29	
		glucose	1.01	1.63	4.01	
		fructose	1.22	11.3	18.21	
		digest with collagenase (% before glycation)				
		days of incubation				
		sample	0	7	14	
		control	100	-	95	
		glucose	100	-	87	
		fructose	100	-	44	
0.2M	collagen	Furosine (%)				[198]
			days of incubation			
		sample	0	7	14	
		control	7	6	8	
		glucose	7	187	304	
		fructose	8	19	42	
0.5M	a and g crystallins	after 2 months fructation there is;				[114]
		an increase in periodate positive material, cross-linking, unfolding, aggregation and resistance to denaturation. decreased tryptophan absorbance and fluorescence				
0.025 & 0.25M	RNase	increases in molecular mass as detected by matrix assisted laser desorption, occurs earlier upon fructation compared with glucation.				[115]

#### 1.6.5. *In vivo* fructation

Direct evidence of *in vivo* fructation caused by dietary fructose can be seen in hereditary fructose intolerance (HFI). There are 3 types of this inborn error of metabolism; 1. fructokinase deficiency; 2. aldolase B deficiency; 3. fructose-1,6-biphosphatase deficiency. The first is seen medically as benign since it only leads to high serum fructose concentrations and fructosuria, however, the fructation occurring in these patients remains to be revealed. Aldolase B reversibly splits fructose-1-phosphate to D-glyceraldehyde and dihydroxyacetone phosphate. People with a deficiency in aldolase B suffer gastrointestinal discomfort upon ingestion of foods containing fructose, continued eating causes hypoglycaemia and can be lethal, patients are treated with a fructose free diet. The third prevents gluconeogenesis which can be a problem with continued fructose ingestion but the main problem is lactic acidosis and uricaemia [96]. Bohles *et al* (1987) reported increased HbA<sub>1ab</sub> in HFI patients of the second variety of inborn error. The concentration of fructated haemoglobin was similar to that of glucated Hb found in diabetics, the authors attributed this finding to the small amount of fructose that is always present in a diet that is supposed to be free of it [150 and 116].

Burden (1984) reported a case of a juvenile IDDM with good glycaemic control yet high glycated haemoglobin. When reducing substances were tested for in her urine, fructose was found. The patient regularly ate diabetic foods that use fructose as a sweetener, substitution of these foods with other carbohydrates (other than glucose) brought the glycated haemoglobin within the normal range within 2 months without affecting her glycaemic control [151].

Direct evidence of *in vivo* fructation, where it is probable that the fructose was derived from the polyol pathway was reported by Walton, McPherson and Shilton

(1988) and (1989). Ten to twenty percent of glycated human ocular lens protein was fructated. The group first showed the production of N-(2-deoxyhexitol-2-yl)lysine (2HL) from fructation and N-(1-deoxyhexitol-1-yl)lysine (1HL) from glucation. These deoxyhexitols, which are derived from borohydride reduction of Heyns and Amadori products were identified using HPLC and spiking with synthetic 1HL and 2HL [88 and 200]. The lens contains no cells only an outer epithelial layer, therefore the source of the fructose for production of Heyns product, remains to be elucidated.



## 1.7. 3-Deoxyglucosone

An alternative hypothesis for the formation of AGEs from fructose was proposed by Shin *et al* (1988). This involved the production of the dicarbonyl compound 3-deoxyglucosone (3-DOG). The basis of this hypothesis was the discovery that fructose at pH 5.5 and 100°C produces 3-DOG. Although these optimal conditions are non-physiological, Shin *et al* (1988) argued that there will be some 3-DOG production from fructose at physiological pH and temperature [112].

Igaki *et al* (1990) reported that proteins incubated with 3-DOG produce the same types of AGE as monosaccharides [186]. Thus 3-DOG is a possible agent for development of AGEs, that is free in solution and highly reactive. Shin *et al* (1988) reported that fructose cross-linked succinylated lysozyme, whereas glucose did not, using arginine residues rather than the blocked lysine residues. The reaction of fructose with the side chain of arginine residues is negligible, thus 3-DOG was proposed by Shin *et al* (1988) to be the cross-linking agent involved, especially because it had been detected after incubation of monosaccharides with native protein. Ames (1993) reported that 1,2-enolisation of Amadori product produces 3-DOG, which reacts with primary amines and Amadori product to produce pyrrolaldehydes which rearrange to form bispyrroles [212]. Figure 1.7.1. is a summary of the reactions of 3-DOG, reproduced from Ames (1993) [212].

3-DOG is likely to be an important post-Amadori intermediate to AGE formation because of its occurrence *in vivo*. Niwa *et al* (1993) reported that the concentration of serum 3-DOG in diabetics was twice that of normal individuals and 4 times in diabetics with nephropathy [119]. *In vivo* metabolism of 3-DOG produces the less reactive compound 3-deoxyfructose (3-DOF) [118]. Knecht *et al* 1992 reported that the concentration of 3-DOF in fasting human plasma was some six times higher

than 3-DOG [118]. The same group in 1994 reported that diabetics had roughly double the concentration of 3-DOF in their urine than normal controls [120].

The rearrangement reactions of Amadori product and fructose, to form 3-DOG, are not redox reactions; yet protein glycated (using glucose, fructose or other monosaccharides) in the presence of a metal chelator, produces virtually no cross-linking. Note that metal chelation did not inhibit cross-linking mediated by 3-DOG under the same conditions [206]. The results of the article by Prabhakaram and Ortwerth (1994) indicate that if 3-DOG is produced in any quantity from Amadori product and fructose, then it requires free metal to form [206].

3-DOG is no doubt an important compound in the production of AGEs. The amount of its contribution to AGE formation from fructose is, however, open to question. Shin *et al* (1988) reported that 3-DOG was produced some 1.3 to 2 times faster in a fructose protein solution than a glucose protein solution. AGE-cross-linking of the proteins in the same solutions was, however, 6 to 7 times higher for fructation of lysozyme than glucation; similarly 15 times for BSA, 2 to 3 times in RNase and 2 fold in ovalbumin [112]. The relative contribution of 3-DOG to AGE formation will vary with the sugar and protein in question and in certain systems this route may only be minor.

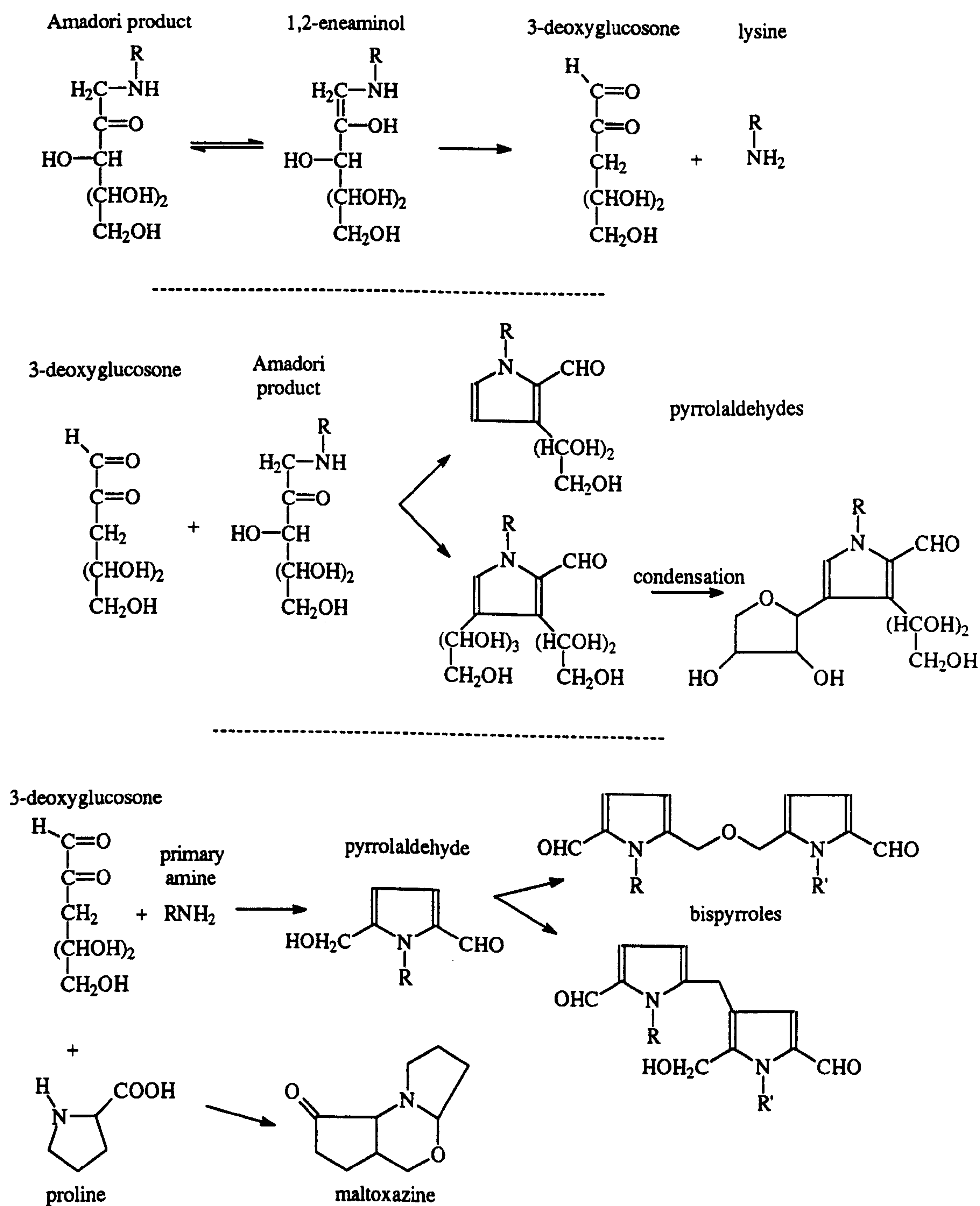


Fig. 1.7.1. Reactions of 3-deoxyglucosone, adapted from Ames (1993) [212]. Note that the production of maltosazine is by reaction with the free amino acid proline, not with a residue of proline in a protein.



## 1.8. A brief review of the proteins used in this study

The model proteins used in this study include serum albumin (human and bovine), lysozyme,  $\beta$ -lactoglobulin and RNase. A thorough review of previous studies of the Maillard reaction using these proteins was published by Cox (1991) and only a brief review is included here [121].

Amadori rearrangement is an acid catalysed reaction so the sites of glycation on the various proteins are principally or initially on lysine residues with a low pKa, or in sequences near side chains that act as proton donors. In serum albumin Iberg and Fluckiger (1986) noted that the most reactive sites were in Lys-Lys, Lys-His, Lys-Lys-Lys and Lys-His-Lys sequences [122]

Serum albumin is the major blood protein in humans; it is found *in vivo* at a concentration that decreases with age but with a mean of about 45mg ml<sup>-1</sup> [127]. Serum albumin is rich in lysine, arginine, cysteine and aspartic acid and has a low number of tryptophan and methionine residues. The high content of cysteine residues is reflected by 17 disulphide bridges that maintain the high stability of the 3 repeat domains that make up the molecule. Serum albumin is an important transport protein of inorganic cations, organic anions, drugs, amino acids, bilirubin haemin and fatty acids which are, in the majority, hydrophobic ligands.

The three repeat domains of serum albumin form an ellipsoid structure with 53% of its secondary structure in the form of  $\alpha$ -helix. The secondary structure of albumin is very sensitive to glycation; one mole of 2-amino-2-deoxyglucose adduct per mole of human serum albumin reduces its  $\alpha$ -helix content to 32% [123]. There are 59 lysine residues in the sequence of serum albumin of which 10 have been shown to become glycated. *In vivo* the principle site of glycation is Lys-525 (33% of

total reaction) while *in vitro* it is Lys-199; the difference is likely to be due to the fatty acids hydrophobically bound around Lys-199 *in vivo*. The fatty acids sterically hinder glycation *in vivo* but it is very likely that they are inadvertently removed during purification [124]. Lys-199 and 525 are thought to be the favoured sites of glycation because of the low pKa that surrounds these sites, this catalyses Amadori rearrangement shifting the equilibrium of glycation away from dissociation of Schiff's base.

**Lysozyme** is an anti-bacterial enzyme found widely in nature; it cleaves the  $\beta(1-4)$  linkage between N-acetylmuramic acid and N-acetylglucosamine, the monomeric constituents of bacterial cell walls [128]. Within the 129 residues of lysozyme are 6 lysines, all of which occur on the external surface with two in sequence (Lys-96 and 97).

Lysozyme has been used as a model for the study of the Maillard reaction mainly by the Kato group in Japan. Cox (1991) reviews their work more thoroughly than in the remit of this thesis [121]. The Kato group have concentrated on cross-linking of lysozyme and other proteins using various sugars; they report that such cross-linking is mediated by free carbonyl compounds (other than the monosaccharides) generated during the course of the Maillard reaction. 3-Deoxyglucosone was identified as a major carbonyl compound generated in a protein sugar mixture and the group have proposed that this is the main cross-linking agent [112].

**$\beta$ -lactoglobulin** is an abundant milk protein that exists *in vivo* in various hydrophobically bound oligomeric states depending on pH, temperature and concentration. It is a  $\beta$ -barrel protein with a strongly hydrophobic interior, this is utilised to transport retinol and fatty acids, although its exact function is unknown [129 and 130]. The protein contains 162 residues with 15 lysine residues; there are



two Lys-Lys sequences at 69, 70 and 100, 101 that could catalyse Amadori rearrangement [125].

The study of  $\beta$ -lactoglobulin in relation to the Maillard reaction has been mostly analysis of the  $\beta$ -lactoglobulin-lactose adduct found in milk, especially stored or processed milk. Lactose is bound to  $\beta$ -lactoglobulin via the glucose residue leaving the galactose moiety free; this forms a powerful antigen that is proposed to be one of the most common food allergens. Five such antigenic sites on the  $\beta$ -lactoglobulin-lactose adduct have been identified and all occur on surface lysine residues [121].

Hitz and Dain (1988) reported that  $\beta$ -lactoglobulin incorporated an unusually high number of sugar residues per mole of lysine as other proteins. This occurred even though the protein did not incorporate as many moles of sugar residues than other proteins [126]. There was no explanation by Hitz and Dain 1988 for the results for  $\beta$ -lactoglobulin, but they could be due to the involvement of amino acids other than lysine in the Maillard reaction as proposed by Shin *et al* (1988) [112]. Alternatively, addition of more sugar residues to the lysine-sugar adduct, possibly by aldol condensation, is occurring; such a mechanism was proposed by Suarez (1991) for the production of pyrroles [87].

**Ribonuclease** (RNase) cleaves RNA and is a single polypeptide of 124 amino acids containing 10 lysine residues. The three most reactive lysine residues (1, 7 and 41) have a low pKa and lie near the phosphate binding region of the active site; the bound phosphate has been reported to catalyse Amadori rearrangement [138 and 139]. McPherson *et al* (1988) reported that RNase incorporated glucose and fructose at the same rate over the first few days of exposure, yet fructose clearly produced more AGEs [88].



## **1.9. Aims and objectives of this thesis**

The overall aim of the work presented in this thesis is to investigate fructation. There are, however, a number of particular points that need to be addressed.

1. To clarify the connection between rates of incorporation and subsequent AGE formation.
2. To compare AGE formation from glucation and fructation.
3. To develop an assay for fructation that will quantify it on a molar basis.
4. To use the fructation assay to elucidate the Maillard reaction as it pertains to fructose.
5. To develop a model to illustrate the cross-linking between a fructated and non-glycated protein in the absence of free sugar. Thus to mimic fructation *in vivo*.

## **2. Methods**

All chemicals were purchased either from BDH laboratory supplies Ltd, Poole, U.K. or Sigma Chemical Co. Ltd., Dorset, U.K. Radiolabels were purchased from Amersham International plc, Amersham, Bucks, U.K.

### **2.1. Buffers**

The water used in all experiments presented in this thesis was equivalent to double distilled, purified using a Elgastat option 3 ultra high quality (UHQ) water purifier. Elgastat quote that no ion or free metal in its UHQ water has a concentration greater than 0.2 $\mu$ g/l; free copper iron have concentrations of <0.01 and <0.02 $\mu$ g/l respectively. All buffers contained 3mM azide as a bactericide and were made up to pH7.4 (adjustment of the pH was either with the salts themselves or with HCl and NaOH). Unless otherwise stated the buffer used was 0.1M sodium phosphate, other buffers used were 10mM HEPES, 10mM TES and 0.5% (w/v) ammonium bicarbonate.

### **2.2. Proteins**

The proteins used in this thesis include; serum albumin (fraction V), hen egg lysozyme, bovine milk  $\beta$ -lactoglobulin and bovine pancreas ribonuclease A. The proteins were purchased from Sigma at various levels of purity; 95% pure lysozyme also contains ovalbumin (45kD) and an unknown protein of a molecular weight of ~73kD. 98% pure  $\beta$ -lactoglobulin contained two variants, A and B, which differ only by two amino acids;  $\beta$ -lactoglobulin also contained a covalently linked dimer, probably produced as a result of Maillard type cross-linking by lactose. RNase A (type X11-A, >99% pure), essentially protease and salt free was

used in the radiolabelling experiments. Serum albumin (human and bovine) was purchased as either 96 to 99 % pure or essentially globulin free (>99% pure); the less pure form contained many serum globulins, mostly of a molecular weight lower than the 66kD of serum albumin, removal of these contaminants was by agarose electrophoresis to produce the 99% pure form. Delipidated serum albumin was also purchased from Sigma. The method used to delipidate the protein used charcoal to avoid denaturation, the same method was also used in some experiments to remove lipid from glycated albumin [171].

### **2.3. *In Vitro* glycation**

Preparation of AGEs was carried out by glycating the various proteins described in section 2.2. Protein and sugar were separately made up to double concentration in the appropriate buffer and mixed together in equal volumes. The protein-sugar mixture was then incubated in the dark (to prevent photo-production of free radicals) in a Memmert universal oven (model UM 100/1) at 37°C. Aliquots were removed periodically and frozen at less than -20°C. The pH of the incubation buffer was checked at intervals to ensure it did not change.

### **2.4. Removal of unbound sugar**

All manipulations of non-frozen, non-diluted sample were carried out on ice. Removal of unbound sugar was carried out at 4°C in a cold room or refrigerator.

Micro-dialysis was carried out using a Bethesda research laboratory (Life Technologies Inc. Gaithersburg MD 20877 USA) micro-dialysis kit with either 6-8kD or 12-14kD cut-off membranes. Exhaustive dialysis was performed against either phosphate or bicarbonate buffers over 4 days with 8 changes of dialysis



buffer and each change of buffer represented a dilution of at least one hundred fold.

**Size exclusion chromatography** combined with ultrafiltration was used in one experiment for very rapid removal of free sugar. Sephadex G25 was hydrated, for 2 hours, with phosphate buffer and then packed into a column (1cm by 10cm). The total, elution and void volumes were calculated from calibration using 10mg/ml haemoglobin in phosphate buffer. 200µl of fructated lysozyme was applied to the column and the 500µl fraction that corresponded to the protein peak was collected. Amicon Microcon concentrators (10kD cut off) were used to reduce the 500µl eluted fraction to 50µl (1 hour, 14000g at 4°C). The microconcentrators also had the effect of further removing free sugar, 90% removal of small molecules is claimed by the manufacturers [131]. The concentrated samples were then diluted with a solution of β-lactoglobulin, in phosphate buffer, to give a final protein concentration of 10mg/ml for both proteins.

## **2.5. Sugar-free re-incubation**

After removal of free-sugar from glycated protein a second native protein was added, either as lyophilised powder, such that the final concentration of the two proteins was equal. Alternatively if the glycated protein had been concentrated during removal of free sugar, it was diluted into buffer containing the native protein, again the final concentrations of the proteins were equal. The two protein (glycated and native) were then re-incubated at 37°C in the Memmert universal oven. A second native protein was introduced in buffer diluted to an appropriate concentration such that both proteins were of known concentration. Aliquots were removed at appropriate intervals, or the whole sample was removed after a set time, and stored at -20°C until needed for assay.

## 2.6. Measurement of protein concentration

Protein concentrations were measured in duplicate using the micro method kit from Pierce. The assay is based on the co-ordination of copper with protein using the method of Smith *et al* (1985) [132]. Briefly, glycated protein was separated from free sugar, which interferes with the assay, and diluted to approximately  $1\text{mg ml}^{-1}$ . To  $20\mu\text{l}$  of standard ( $0$  to  $2\text{mg ml}^{-1}$ ) or sample on a microplate, was added  $200\mu\text{l}$  of detection reagents A and B (see below for recipe) which were mixed in a ratio of 50:1. The microplate was covered and samples vortexed on a Wellmix 1 microplate stirrer before 30 minutes incubation at  $37^{\circ}\text{C}$ . The protein concentration of the samples were calculated from absorbance at  $570\text{nm}$  (a typical calibration graph is illustrated in figure 2.6.1.).

Contents of detection reagents as reproduced from Smith *et al* (1985) [132]: Reagent A, 1% disodium salt of bicinchoninic acid, 2%  $\text{Na}_2\text{CO}_3\cdot\text{H}_2\text{O}$ , 2% disodium tartrate, 0.4%  $\text{NaOH}$ , 0.95%  $\text{NaHCO}_3$  with further addition of the last two to adjust the pH to 11.25: Reagent B, 4%  $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ .

When appropriate, the protein concentration of serum albumin was measured using its absorbance at  $280\text{nm}$  on a Cecil 4000 spectrophotometer. The assumption was made that  $1\text{mg ml}^{-1}$  of non-glycated serum albumin had an absorbance of 0.67 at  $280\text{nm}$  when read against distilled water (the assumption was also checked using the Pierce protein assay). The production of AGE chromophores on diluted glycated protein was measured in a similar manner using a range of UV wavelengths.

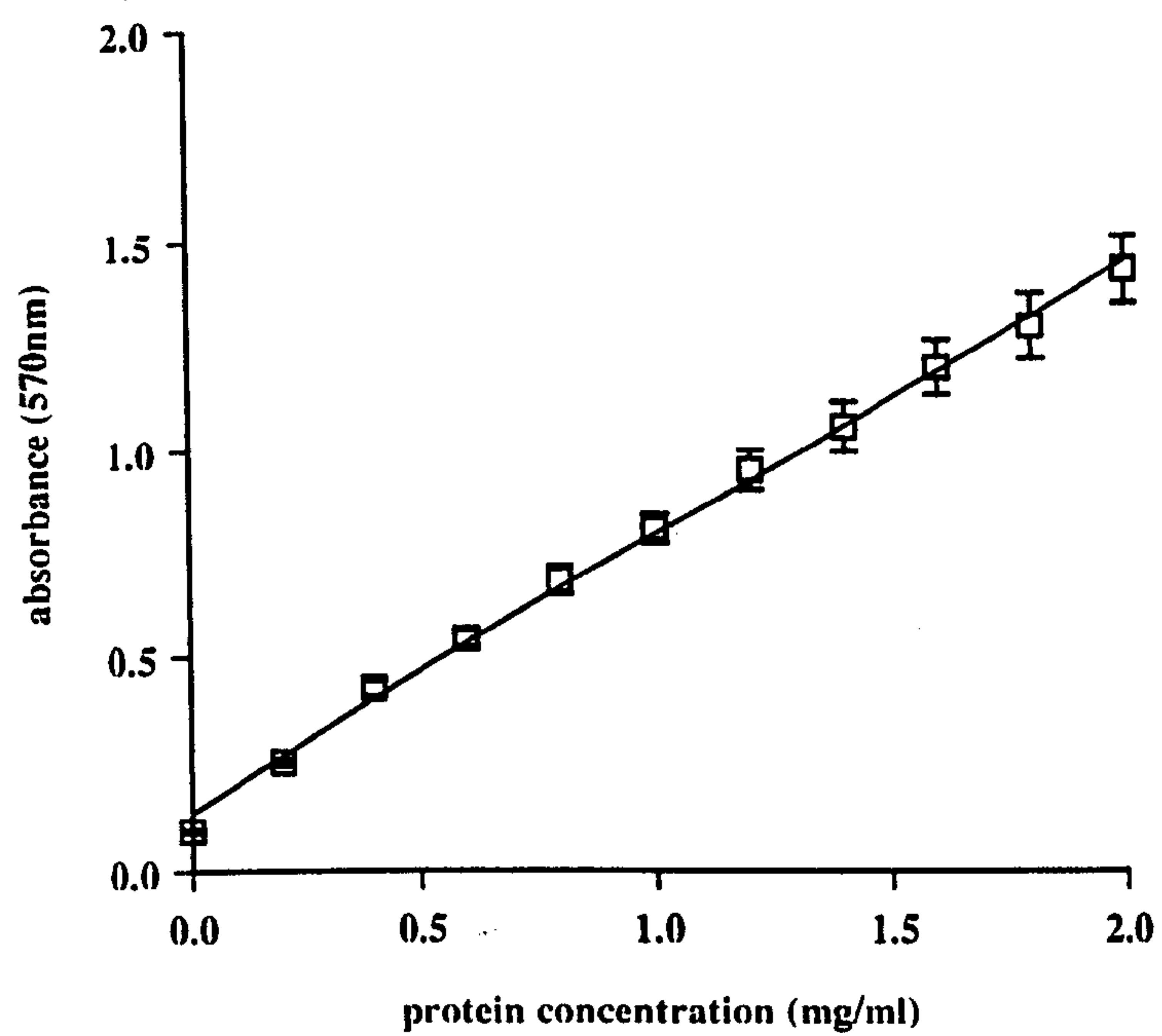


Fig. 2.6.1. Typical calibration curve for the Pierce protein assay using BSA.



## **2.7. Periodate assay**

Periodate positive material, including Amadori product was measured by periodate oxidation, according to the micro-assay method of Ahmed (1992); the protocol of which is as follows [133];

1. Prepare fructose calibration standards from 0 to 60nM in the same buffer as the sample (usually bicarbonate buffer).
2. In duplicate pipette 60 $\mu$ l of sample (10mg ml<sup>-1</sup> of protein) or fructose standard to 1.5ml eppendorfs;
3. Add 30 $\mu$ l of 0.1M HCl, followed by 30 $\mu$ l of 0.05M NaIO<sub>4</sub>, vortex and allow to react at room temperature for 30 minutes.
4. The reaction is stopped by placing on ice and adding 30 $\mu$ l of pre-cooled 0.7M NaOH, followed by 30 $\mu$ l of pre-cooled 15% ZnSO<sub>4</sub> (w/v)
5. Vortex and centrifuge at 14000g for 10 minutes to form a pellet of the precipitate.
6. Remove 100 $\mu$ l of supernatant to a microplate and add 200 $\mu$ l formaldehyde detection reagent (10ml 3.3M ammonium acetate with 46 $\mu$ l acetyl acetate).
7. Cover the microplate and incubate for 1 hour at 37°C.
8. Measure the absorbance at 405nm. I used a Bio-Tech microplate autoreader (model EL311, Luminar Technology, Hampshire).

## 2.8. Fluorescence

Fluorescence was measured on an Aminco Bowman fluorimeter, model J4-8960, or a Perkin Elmer fluorimeter, model LS50B, (spectral band width: 10nm for both excitation and emission). Calibration of both machines was carried out using 1ng ml<sup>-1</sup> of quinine sulphate made up in 0.1M H<sub>2</sub>SO<sub>4</sub>. All measurements were made in at least duplicate, the results were corrected for any daily changes in the fluorescence readings of the quinine sulphate standard and expressed as relative fluorescence per mg of protein.

Fluorescence was measured at the wavelengths that correspond to the maximum for the AGE biomarker pentosidine (excitation 325nm and emission 375nm) or that attributed to the maximum for glycated BSA (excitation 350nm and emission 420nm) [14]. The concentration of protein used for the fluorescence measurements was never more than 1mg ml<sup>-1</sup>, such that the UV absorbance did not exceed more than 1 and quenching of the fluorescence was reduced to minimum.

Fluorescence spectra were measured by either exciting at a single wavelength and measuring the emission spectra or *vice versa*.

## 2.9. SDS-PAGE

Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Laemmli 1970 [135] on Bio Rad minigels (8 x 10cm, 1mm thick). The constituents of the gel, unless otherwise stated, were;

**Resolving gel:** 15% (w/v) acrylamide, 0.4% (w/v) PDA, 0.75M Tris-HCl pH8.8, 0.1% (w/v) SDS, 0.5% (w/v) ammonium persulphate and 0.5% (v/v) TEMED all made up in ultra pure water. The gel was topped with water to exclude atmospheric oxygen (polymerisation is inhibited by oxygen) and poured off after the gel had set. Any remaining water was removed with a corner of tissue before layering of the stacking gel.

**Stacking gel:** 4% (w/v) acrylamide, 0.1% (w/v) PDA, 0.124M Tris-HCl pH6.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate and 0.1% (v/v) TEMED all made up in ultra pure water. A comb was inserted to obtain sample wells.

**Sample preparation:** 50  $\mu$ l of protein sample, diluted to 1mg ml<sup>-1</sup> with appropriate buffer, was boiled for 5 minutes with 25 $\mu$ l of freshly made sample buffer; 200mM Tris-HCl pH6.8, 10% (v/v) glycerol, 5% (w/v) SDS, 0.01% (w/v) bromophenol blue and 5% (v/v) 2-mercaptoethanol.

**Molecular weight markers:** Markers ranging from 14 to 66 kD were re-constituted in phosphate buffer and underwent the same preparation as the samples before loading on to the gels. The markers were obtained as a pre-mixed lyophilised vial (code; SDS 6) from Sigma, Pool, Dorset, UK.



**Electrophoresis:** Once the stacking gel had set, the minigel apparatus was assembled and running buffer (25mM Tris base, 192mM glycine and 0.1% (w/v) SDS at pH8.3) poured over the top and bottom of the gel to complete the circuit. 10µl of freshly boiled sample or marker were applied to the wells of the stacking gel. Once all the samples or markers had been loaded, the apparatus was connected to a Bio Rad power supply, 170V was applied until the bromophenol blue was near the bottom of the resolving gel (about 1 hour).

### 2.9.1. Gel staining

Gels were either stained with colloidal Coomassie brilliant blue (CCBB) or Bio Rad silver stain plus.

**CCBB staining:** After electrophoresis the gels were removed to a single dish and stained overnight according to the optimal one step method of Neuhoff (1988), with additional destaining in the storage solution (20% ammonium sulphate) overnight [136]. This method has the advantage over conventional Coomassie staining in that it utilises the colloidal properties of the stain; the fixing, staining and destaining steps are all combined in a single step. A second advantage of this method is its sensitivity, the method has been reported to detect as little as 0.5-1ng of protein compared to other conventional Coomassie brilliant blue methods that have a detection limit of around 0.2-0.5µg of protein [136]. The only drawback of the method is that the stain has to be made fresh each time otherwise the Coomassie brilliant blue precipitates out and the colloidal properties are lost.

The protocol;

1. Overnight staining, on a rocking table, in 0.1% (w/v) Coomassie brilliant blue, 2% (v/v) phosphoric acid, 10% (w/v) ammonium sulphate and 20% (v/v) methanol. The stain was most easily prepared if the Coomassie

brilliant blue was dissolved in the methanol and the ammonium sulphate in water before mixing the two together with phosphoric acid.

2. Brief wash of the gels and staining vessel with 25% (v/v) methanol to remove adsorbed CCBB stain.
3. Overnight destaining in storage solution (20% ammonium sulphate, w/v).

**Bio Rad silver stain plus:** After electrophoresis, gels were removed to separate vessels that had been cleaned with 50% (v/v) nitric acid and rinsed with water. The gels were then stained according to the manufacturer's instructions [137].

Figure 2.9.1.1. compares the CCBB stain with that of Bio Rad silver stain plus, both of these stains have similar sensitivities (0.5-1.0ng of protein per band) but give slightly different staining patterns [137]. Samples of a solution of lysozyme and  $\beta$ -lactoglobulin in phosphate buffer were incubated at 37°C for 7 days with and without 0.1M fructose. The two samples were then prepared in sample buffer and loaded (using 13.4 $\mu$ g of protein in 10 $\mu$ l of sample buffer per lane) onto two 15% SDS polyacrylamide gels; after simultaneous electrophoresis one of the gels was stained with the CCBB stain and the other with silver. If the sugar-free controls (lanes A and C in Fig. 2.9.1.1.) are regarded as the standard stain for these proteins, lanes B and D illustrate the effect of 7 days fructation on the stain. Some loss of monomer with fructation would be expected due to cross-linking, however silver staining indicates almost complete loss of  $\beta$ -monomer (lane B), while CCBB staining of the same sample (lane D) does not indicate any such loss. In contrast, fructated lysozyme is less well defined in the CCBB stain than the silver. The silver stain also fails to stain the high molecular weight aggregates that are very clear on the CCBB stain. The 28kD lysozyme homodimer and 32kD heterodimer are clear with both stains although the contrast becomes better on the CCBB when it has



been further destained by overnight storage in 20% ammonium sulphate (w/v, results not shown).

The addition of sugar residues to what was a positively charged lysine residue by glycation possibly inhibits binding of SDS and thus complete denaturation of the protein. Incomplete denaturation causes proteins to run as diffuse rather than discrete bands upon SDS-PAGE, thus the greater the extent of glycation the broader the protein band in the gel. Figure 2.9.1.1. illustrates that the Bio-Rad silver stain is also inhibited by glycation. The manufacturers informed us that glycosylated proteins are poorly stained, thus glycation is probably preventing the stain by a similar mechanism.

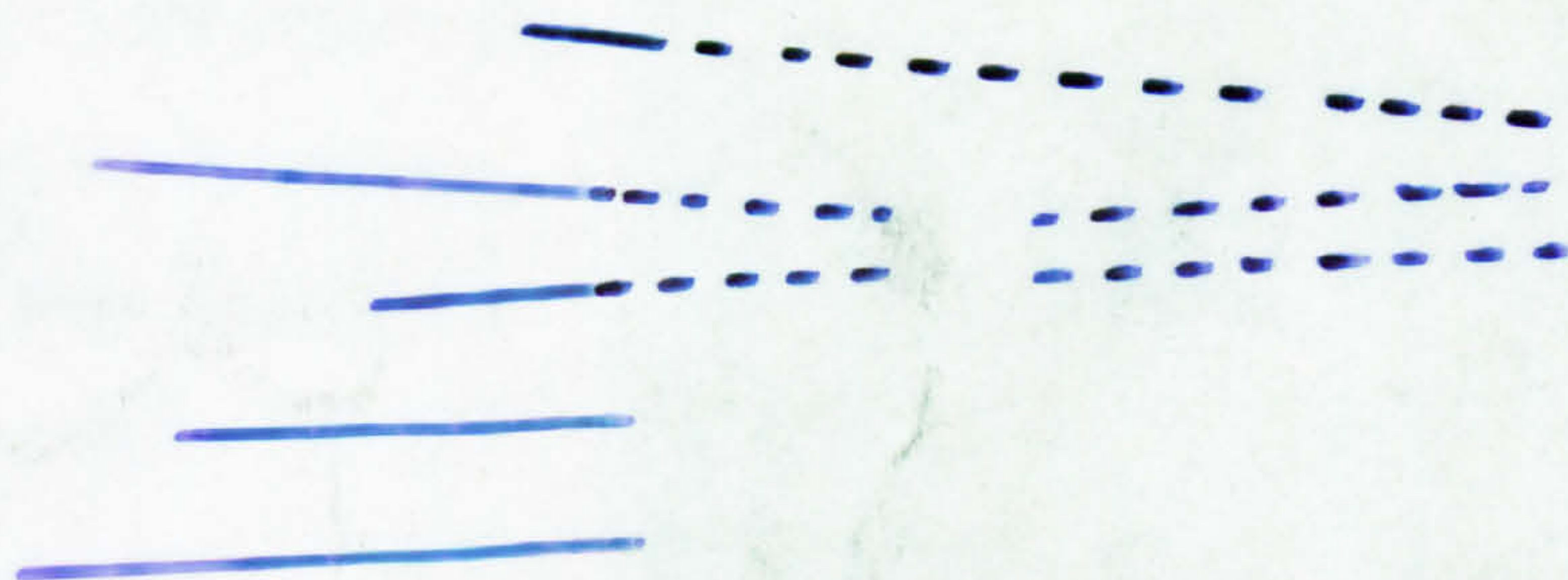
The mechanism of initiation of silver staining is obscure and various amino acids have been implicated; polyhistidine gives the most intense reaction out of all the water soluble amino acids but lysine, cysteine, methionine, aspartic acid and glutamic acid also play a role [202]. It is probable that fructation of  $\beta$ -lactoglobulin sterically hinders the site of silver ion complexation; either by the hexose itself blocking the site, or by inducing a conformational change in the protein that buries the site of complexation. This suggests that the glycation of  $\beta$ -lactoglobulin could be followed by loss of silver staining after SDS-PAGE. Conversely, Hodny *et al* have used silver stain to show glycation of collagen and BSA, they attribute the more intense staining that they have found, as a function of the period of glycation, to silver ion complexation with Maillard reaction products [203 and 204].



CLEAR OVERLAYS USED IN THE ORIGINAL  
THESIS.

Silver state

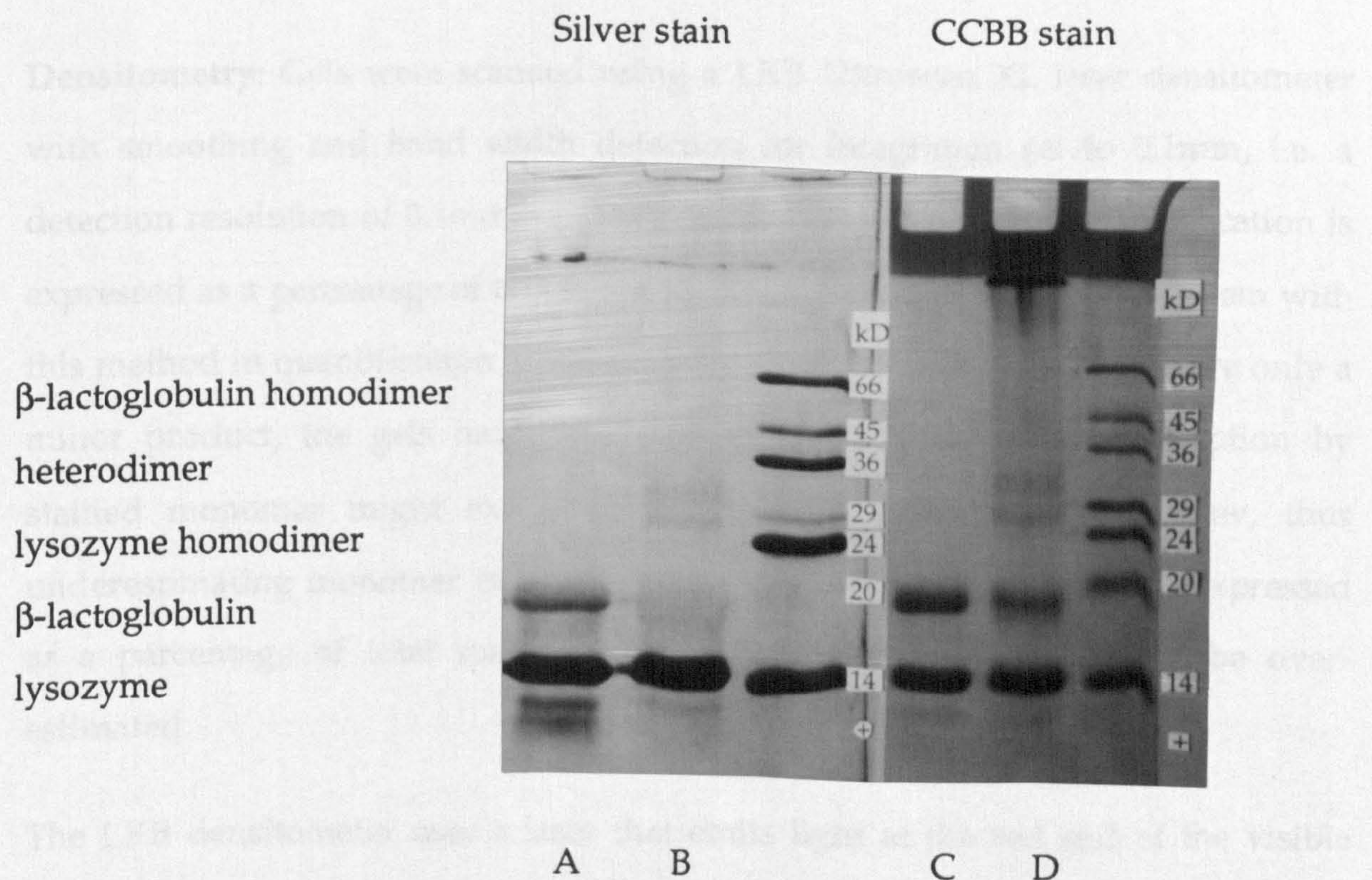
CC 28 - 31





29.1. Photography and Densitometry

Photography: Gels were photographed using a Polaroid Land Camera and type 55 film with various intensities and exposure times used to obtain the best contrast of bands against the background.



The 1.78 densitometer was used to measure the intensity of the visible spectrum and that of the ultraviolet spectrum of the samples. The silver stain was used to measure the intensity of the visible spectrum. The actual wavelengths detected in the silver stain were 400-450 nm. The bands are the same as those seen in the silver stain. The bands are the same as those seen in the silver stain.

Fig 2.9.1.1. A comparison of the staining by colloidal Coomassie brilliant blue and Bio Rad silver stain plus. The control lanes A and C represent 7 days incubation of lysozyme and  $\beta$ -lactoglobulin without free sugar. Lanes B and D included 0.1M fructose during the 7 days of incubation.



## 2.9.1. Photography and Densitometry

**Photography:** Gels were photographed using a Polaroid Land Camera and type 55 film with various apertures and exposure times used to obtain the best contrast of bands against the background.

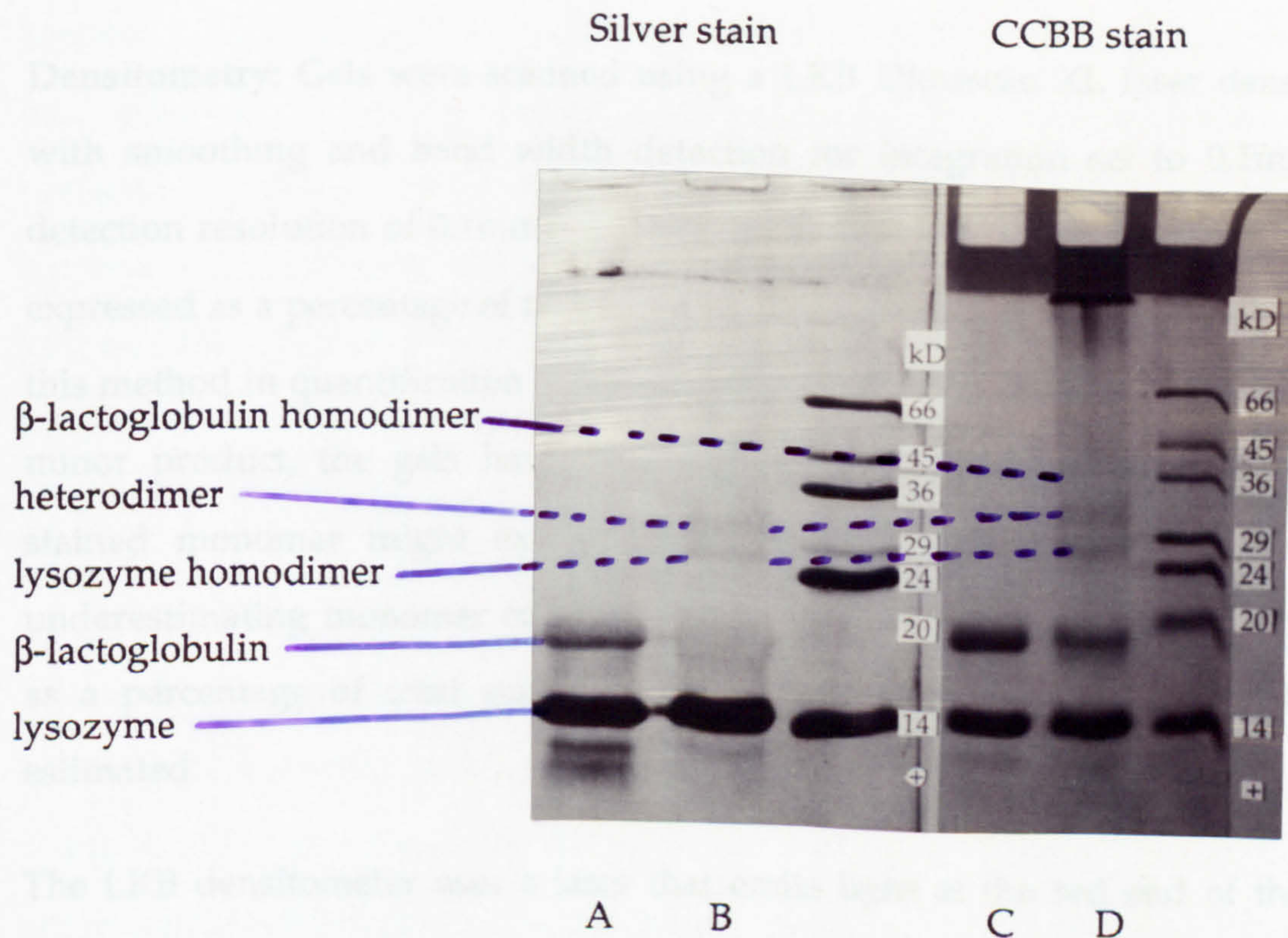


Fig 2.9.1.1. A comparison of the staining by colloidal Coomassie brilliant blue and Bio Rad silver stain plus. The control lanes A and C represent 7 days incubation of lysozyme and  $\beta$ -lactoglobulin without free sugar. Lanes B and D included 0.1M fructose during the 7 days of incubation.



### 2.9.2. Photography and Densitometry

**Photography:** Gels were photographed using a Polaroid Land Camera and type 53 film with various apertures and exposure times used to obtain the best contrast of bands against the background.

**Densitometry:** Gels were scanned using a LKB Ultrosan XL laser densitometer with smoothing and band width detection for integration set to 0.1mm, i.e. a detection resolution of 0.1mm. The amount of oligomer produced by glycation is expressed as a percentage of the total amount of stain in that lane. A problem with this method in quantification is that in order to observe cross-links that are only a minor product, the gels have to be overloaded. Therefore the absorption by stained monomer might exceed the bounds of the Beer-Lambert law, thus underestimating monomer concentration. Because the other bands are expressed as a percentage of total stain for the whole lane these would thus be overestimated.

The LKB densitometer uses a laser that emits light at the red end of the visible spectrum and thus is at the optimum for absorption by Coomassie brilliant blue. The silver stain, however, absorbs in the blue green area of the visible spectrum (actual wavelengths depend on the protein stained), thus poorly stained minor products are also poorly quantified using this densitometer. Since the CCBB stain is as sensitive as the silver and is more readily quantified, the CCBB stain has been used as the standard stain in this thesis unless otherwise stated.

The gels were finally dried using a LKB 2003 slab gel dryer and stored in a low humidity environment.

### **3. Conditions necessary for AGE formation from fructose**

#### **3.1. Introduction**

Fructation is an area that has not previously been extensively studied, partly because it is not detected by the assays normally used to detect glycation [209]. It was therefore necessary, before a more in depth study of fructation was undertaken, to investigate the conditions required for AGE formation from fructose, using methods that can relate it to glycation by other sugars, particularly glucose. This chapter of the thesis investigates the effect of different experimental conditions on a readily available measure of fructation, i.e. fluorescence. The rate of development of fluorescence due to glycation by glucose and fructose is also compared to the rates of incorporation of the two sugars.

I have already reviewed the literature concerning studies of fructation in section 1.6. but a brief recapitulation is necessary here. Throughout the literature fructose produces AGEs at a faster rate than glucose, the actual rates vary according to the experimental conditions and AGE studied. However, the picture of what happens in the Maillard reaction between free fructose and production of AGEs is very confused by the contradictory results published by a number of authors. Suarez *et al* (1989 and 1991) reported that the rate of blockage of primary amino groups of BSA by glucose and fructose was the same; the higher rate of AGE formation from fructose was attributed to the addition of more than one molecule of fructose per lysine residue, with the resultant structure forming a pyrrole [14 and 87]. This hypothesis could be modified considering the work by Shin *et al* (1988) [112]. The similarity in loss of primary amino groups, upon glucation or fructation, may be illustrating similar rates of production of Amadori and Heyns products; the additional AGEs observed upon fructation, compared to glucation, may be due to



enolisation of fructose to 3-DOG which will produce AGEs via arginine. Studies using radiolabelled monosaccharides indicate that fructose forms Schiff's base with haemoglobin some 7.5 times faster than glucose, which tends to indicate that Heyns product would form some 7.5 times faster than Amadori product [83]. Conversely McPherson *et al* (1988) report that HSA incorporated glucose eight times faster than fructose and RNase incorporated both monosaccharides at the same rate [88]. I have repeated the experiment reported by McPherson *et al* (1988) to try and shed some light on the confused picture of fructation painted by the literature [88].

*In vitro* experiments investigating disease processes are usually set up so that the conditions mimic those *in vivo*. Phosphate buffer is used almost exclusively throughout the literature for the study of AGE formation, although the exact conditions vary from paper to paper. Watkins *et al* (1987) reported that the inorganic phosphate complexes with RNase near the site of glycation and catalyses Amadori rearrangement [138]. The same group, in 1992, reported that a component of phosphate buffer was taking part in post-Amadori reactions because increasing the concentration of the buffer increased the rate of AGE formation [133]. The group have since reported that it is the free metal of phosphate and other inorganic buffer that takes part in post-Amadori reactions; if the free metal is chelated or if organic buffers (which have a low concentration of free metal contamination) are used, then AGE formation is inhibited [51, 134, 139, 140, 138 and 140].

In order to produce detectable amounts of AGEs, many *in vitro* studies glycate for long periods of time with high concentrations of sugar. Although such an approach does produce AGEs, there may be reactions occurring that are non-physiological. *In vitro* experiments set up to mimic conditions *in vivo* will produce

results that are more relevant to the pathology of diabetes. This chapter is a good place to show how AGE formation relates to monosaccharide concentration. Several authors have shown that the glucose incorporation into protein and the subsequent AGE formation is directly proportional to the glucose concentration up to 40mM [141, 148 and 185]. Meriesh *et al* (1982) reported similar results up to 0.14M glucose, however interpolation of the points on figure 2, of the article, would have produced an exponential growth curve rather than the least squares fit presented [142]. The results of these articles indicate that above a glucose concentration of 40mM the Maillard reaction is not proceeding at the same rate as below 40mM. Thus experiments using glucose concentrations that exceed 40mM may be investigations of non-physiological reactions. If this hypothesis is true then much of the literature concerning the Maillard reaction *in vivo* could be criticised and said to have been studies of artefacts. The problem of the low yield of AGEs upon glycation with physiological concentrations of sugar has been overcome by a more sensitive assay, namely the study of inactivation of enzymes due to glycation. Ganea (1988) reported decreased activity of several enzymes upon glycation with as little as 5mM glucose over 6 days incubation [65].

The study of production of chromophores upon glycation was largely outside the remit of this thesis because the classic browning products, (melanoidins) of food science are both Maillard and lipid peroxidation products. Note that melanin, the dark pigmentation of skin, hair, etc. is an oxidation product of tyrosine and dihydroxyphenol compounds, and should not be confused with the Maillard reaction. This chapter is however a convenient place to illustrate the changes in absorbance spectra of protein due to glycation. Two types of chromophore are reported in the literature; those that occur in the UV region, which are probably the absorbance of fluorophores and those that occur after production of fluorophores in the visible region [51 and 14].

### **3.2. Aims**

1. To compare the rate of incorporation of glucose and fructose into various proteins.
2. To compare the rate of radiolabelled hexose incorporation with the subsequent rate of development of AGE-fluorescence.
3. To illustrate the effect of various buffers on the development of AGE-fluorescence.
4. To investigate the effect of sugar concentration on development of AGE-fluorescence.



### 3.3. Methods

The incorporation of hexose into protein was investigated by glycation of native HSA, BSA (40mg ml<sup>-1</sup>) and RNase (type X11-A, 20mg ml<sup>-1</sup>) with glucose or fructose (0.1M) that had been spiked with 46μCi of the appropriate [U-<sup>14</sup>C]-monosaccharide. The duplicate samples were incubated at 37°C for 8 days in 0.1M phosphate buffer. At appropriate time points during the 8 days, 25μl of each sample was removed in duplicate. To the 25μl aliquot was added 0.5ml of 10M fructose; this was to compete with the labelled monosaccharide for sites of non-specific adsorption to the protein. The protein was precipitated by addition of 0.5ml of cold 20% trichloroacetic acid (TCA) and centrifugation at 14000g for 5 minutes to form a pellet. The supernatant was pipetted away for appropriate disposal and the pellet resuspended in 0.5ml 0.5M sodium hydroxide. It was necessary to wash the protein by repeated precipitation and resuspension, with TCA and NaOH, 6 times before all excess (non-covalently bound) labelled hexose was removed (results not shown). The final resuspension was in 1ml of 0.5M sodium hydroxide which was transferred to scintillant and the counts per minute measured. The activity of the protein and thus the total moles of sugar (labelled and linear extrapolation to non-labelled) incorporated into that protein were calculated by daily calibration of the scintillation counter with 1ml of radiolabel diluted with NaOH. All radioactivity was accounted for and disposed of according to local rules. The recovery of the protein from the vigorous washing procedure was determined, using non-labelled protein, by measuring the protein concentration, by its absorbance at 280nm and by the Pierce protein assay (page 58), before and after the washing procedure. The percentage recovery of the proteins were approximately 80% for the serum albumins and 10% for RNase. All

results are expressed per mole of protein to take into account any loss of protein, but the low recovery of RNase makes interpretation of the results very difficult.

The incorporation of hexose into protein was compared to the fluorescence (page 62) of samples glycated in an identical manner but without the radiolabel spiking.

**The effect of different buffers (all pH 7.4) on AGE fluorescence** was studied by substitution of phosphate buffer with non-buffered double distilled water, 10mM TES or 0.5% bicarbonate buffers. The results shown are the change in fluorescence that occurred during 7 days fructation.

**The effect of sugar concentration** on development of AGE-fluorescence was investigated using 10mg ml<sup>-1</sup> BSA (fraction V) incubated for 0, 5 and 10 days with various concentrations of glucose and fructose.

**Formation of AGE-chromophores** was investigated by either glyrating BSA (fraction V) with 0.2M glucose for up to 18 days or fructating (0.1M fructose) essentially globulin free HSA (fraction V, ~99% pure) for up to 8 days. The spectra were measured from 200 to 400nm.

**Changes in protein secondary structure** due to fructation were investigated by the changes in the proteins UV absorbance. Fructated lysozyme (10mg ml<sup>-1</sup>) was prepared by; 'minimal fructation,' lysozyme in phosphate buffer was dialysed for 12 hours at 4°C introducing up to 5mM fructose in bicarbonate buffer; or by 24 hours glycation (0.5M fructose, 37°C in phosphate buffer) in addition to the minimal fructation. To the fructated lysozyme was added 10mg ml<sup>-1</sup> β-lactoglobulin and the absorbance spectra of the samples measured before and after 7 days incubation at 37°C. The purpose of this experiment was to illustrate post-Amadori or post-Heyns reactions and emphasise that only a short period of glycation is necessary to produce AGEs.



### **3.4. Results**

#### **3.4.1. Comparison of fructation with glucation**

Figure 3.4.1.1. illustrates the incorporation of glucose and fructose into HSA, BSA and RNase. In HSA and RNase the results indicate a similar rate of incorporation for glucose and fructose up to 3 days of incubation; after 3 days, and at all time points for BSA, fructose is incorporated to a higher degree than glucose. A higher rate of incorporation of fructose is matched by a higher rate of development of AGE-fluorescence in all the proteins upon fructation compared to glucation (Figs. 3.4.1.2. and 3.4.1.3.).

Figures 3.4.1.4. and 3.4.1.5. compare AGE-fluorescence with incorporation of hexose. For up to 15 moles of fructose or 9 moles of glucose incorporation per mole of protein, HSA and BSA both exhibit the same relative fluorescence as a function of incorporation of the sugars. This suggests that because BSA incorporates hexose at a faster rate than HSA, it develops more AGE-fluorescence over the same period of time. Fructated BSA fluorescence forms a plateau with respect to incorporation of fructose after 15 moles per mole (Figs. 3.4.1.4. and 3.4.1.5.). This indicates that incorporation of hexose continues unabated even after development of fluorescence ceases (compare Figs. 3.4.1.1. and 3.4.1.2.). It is probable that the AGE-fluorophores themselves, or their precursors, are taking part in other post-Amadori or post-Heyns reactions to produce other types of AGE.

Figures 3.4.1.6. and 3.4.1.7. show the fluorescence excitation and emission spectra at the two sets of wavelengths quoted in the methods chapter (see page 62). The fluorescence readings have been adjusted for any daily differences in the absolute fluorescence of quinine sulphate (the calibrant) and divided by the protein



concentration. The spectra show that AGE-fluorescence is a very broad single peak rather than several sharp distinct peaks; this implies that there are many fluorophores with overlapping excitation and emission wavelengths. Indeed at the wavelengths used for the biomarker pentosidine, excitation at 325nm produces a peak around 410nm and thus it is the shoulder, at 375nm, that is used to assess pentosidine fluorescence.

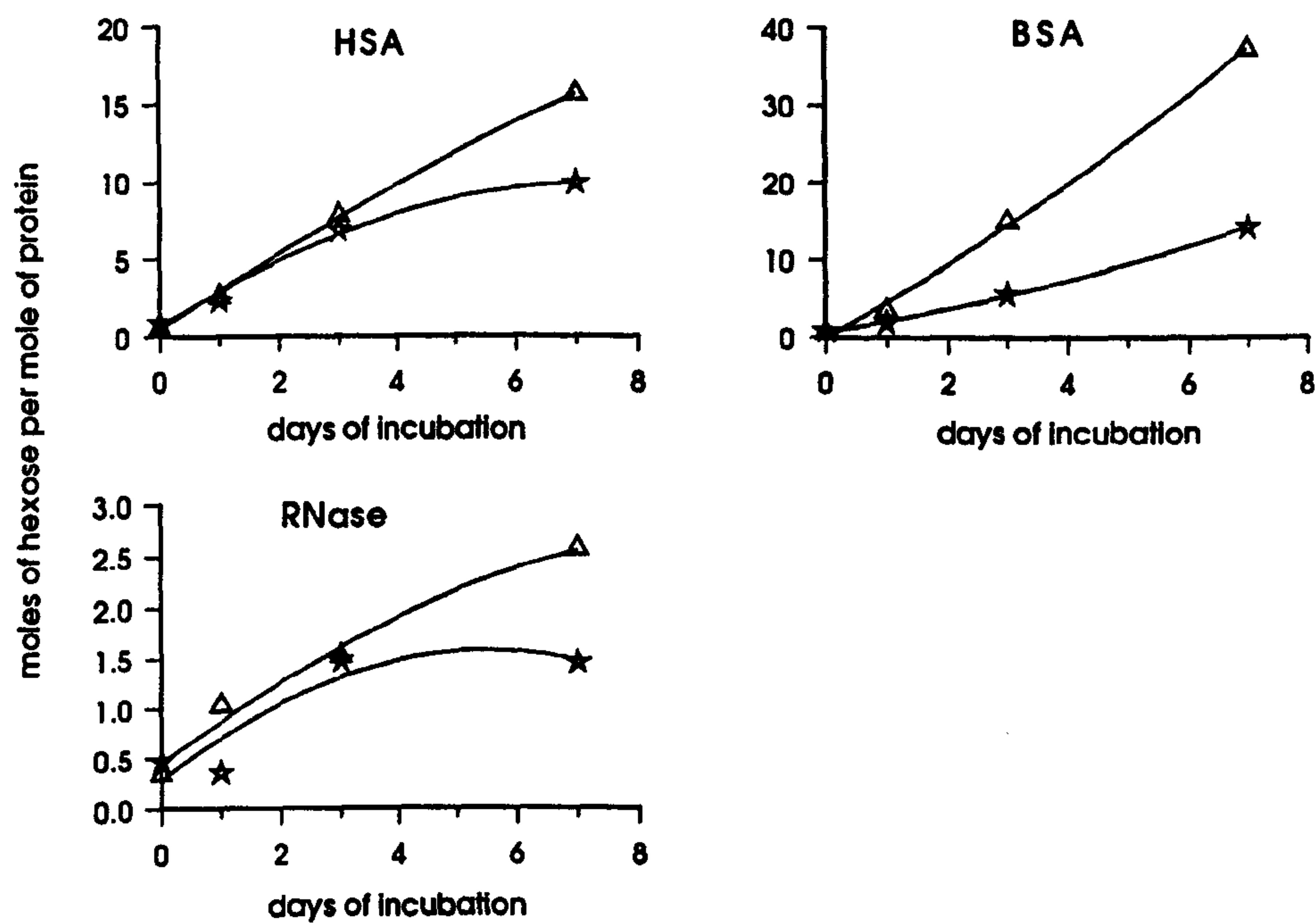


Fig. 3.4.1.1. Incorporation of monosaccharide into HSA, BSA and RNase upon fructation or glucation for up to 7 days. Incubation with 0.1M monosaccharide is indicated by the triangle symbol for fructose and star for glucose.

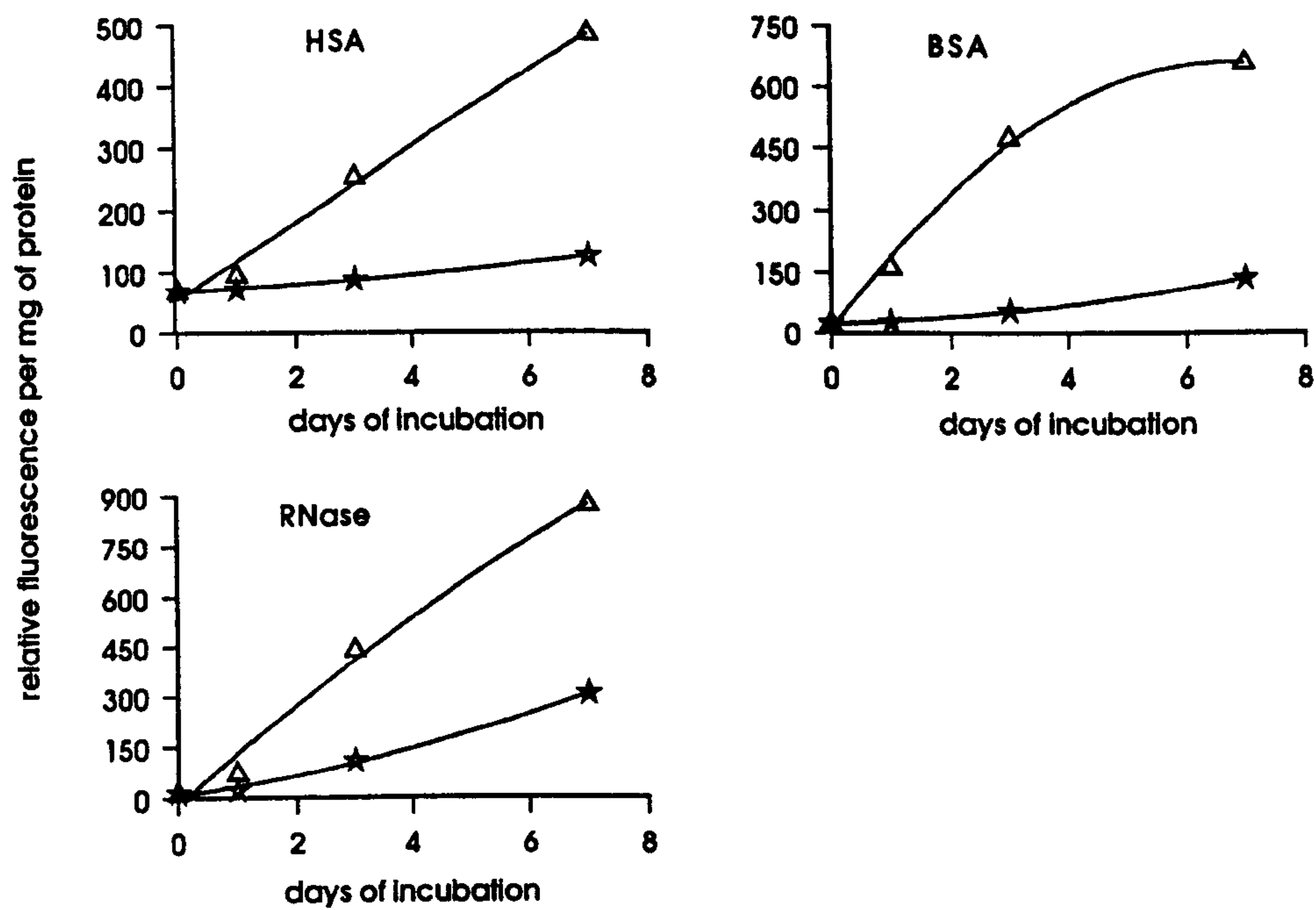


Fig. 3.4.1.2. Pentosidine-linked fluorescence of HSA, BSA and RNase on up to 7 days fructation or glucation. Incubation with 0.1M monosaccharide is indicated by the triangle symbol for fructose and star for glucose.



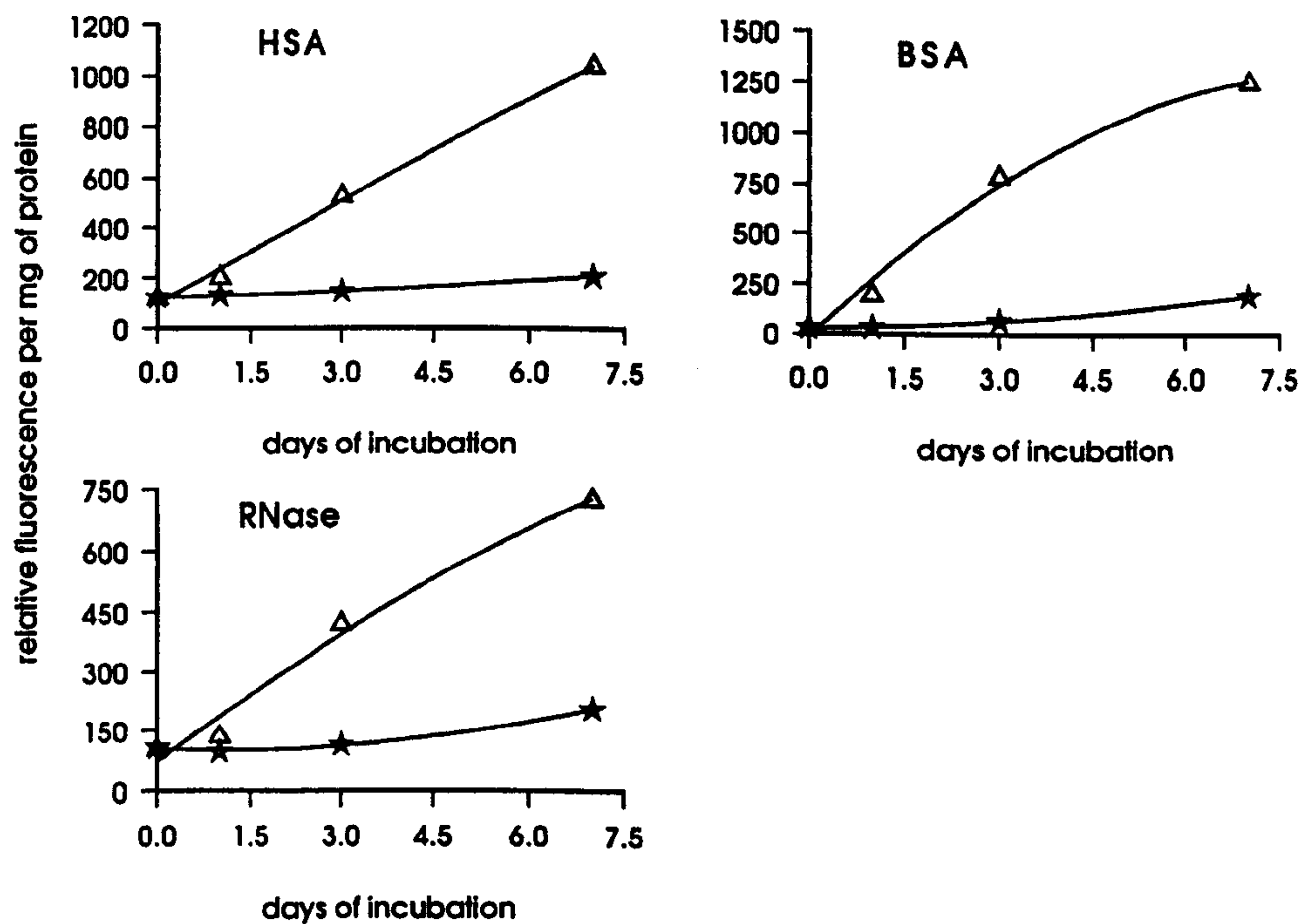


Fig. 3.4.1.3. Fluorescence, at an excitation wavelength of 350nm and emission of 420nm, on up to 7 days fructation or glucation. Incubation with 0.1M monosaccharide is indicated by the triangle symbol for fructose and star for glucose.

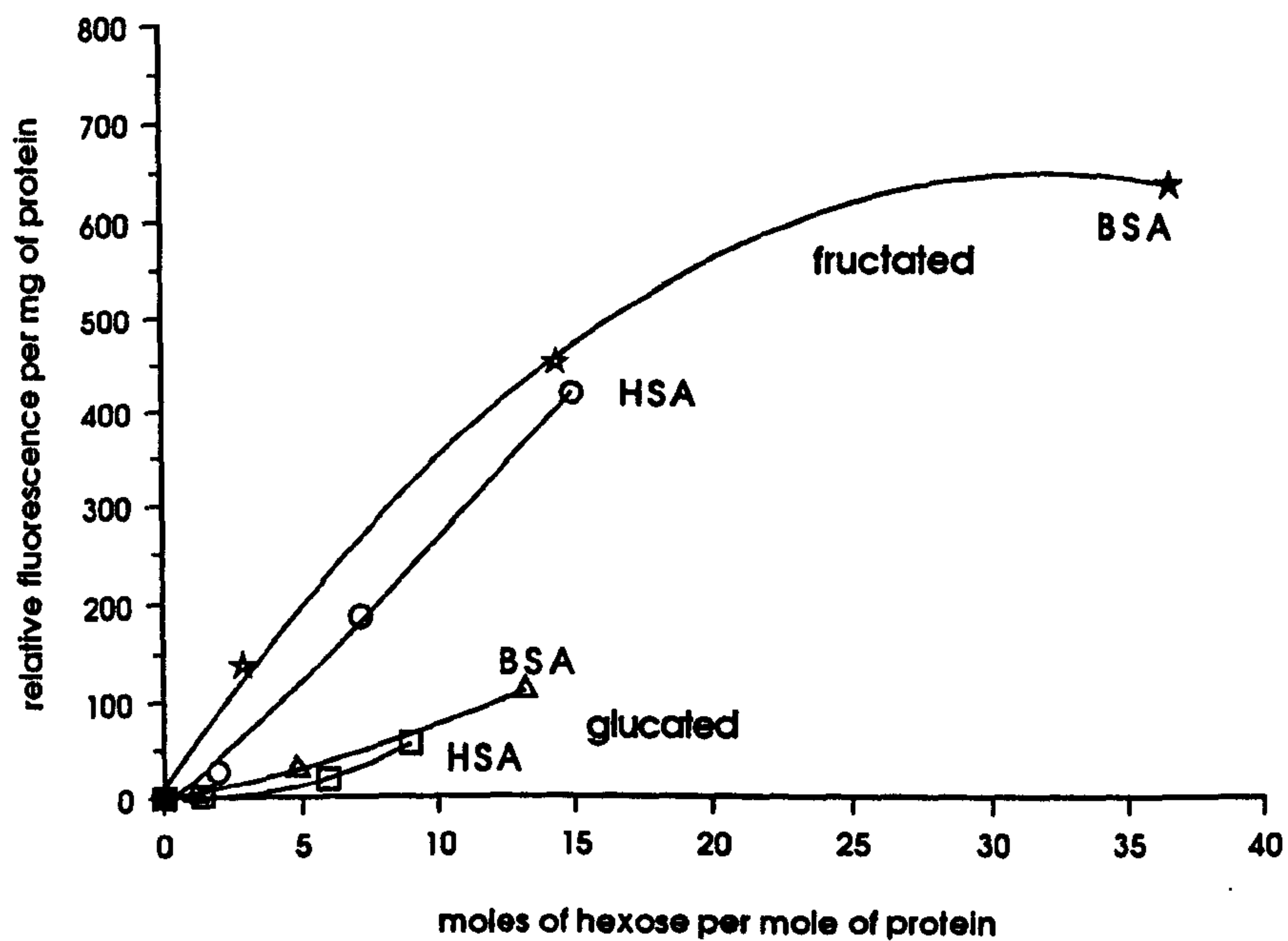


Fig. 3.4.1.4. Comparison of development of pentosidine fluorescence, due to glycation, with the total incorporation of hexose into HSA, BSA and RNase. Proteins were glycated with either fructose or glucose (0.1M) for up to 7 days.

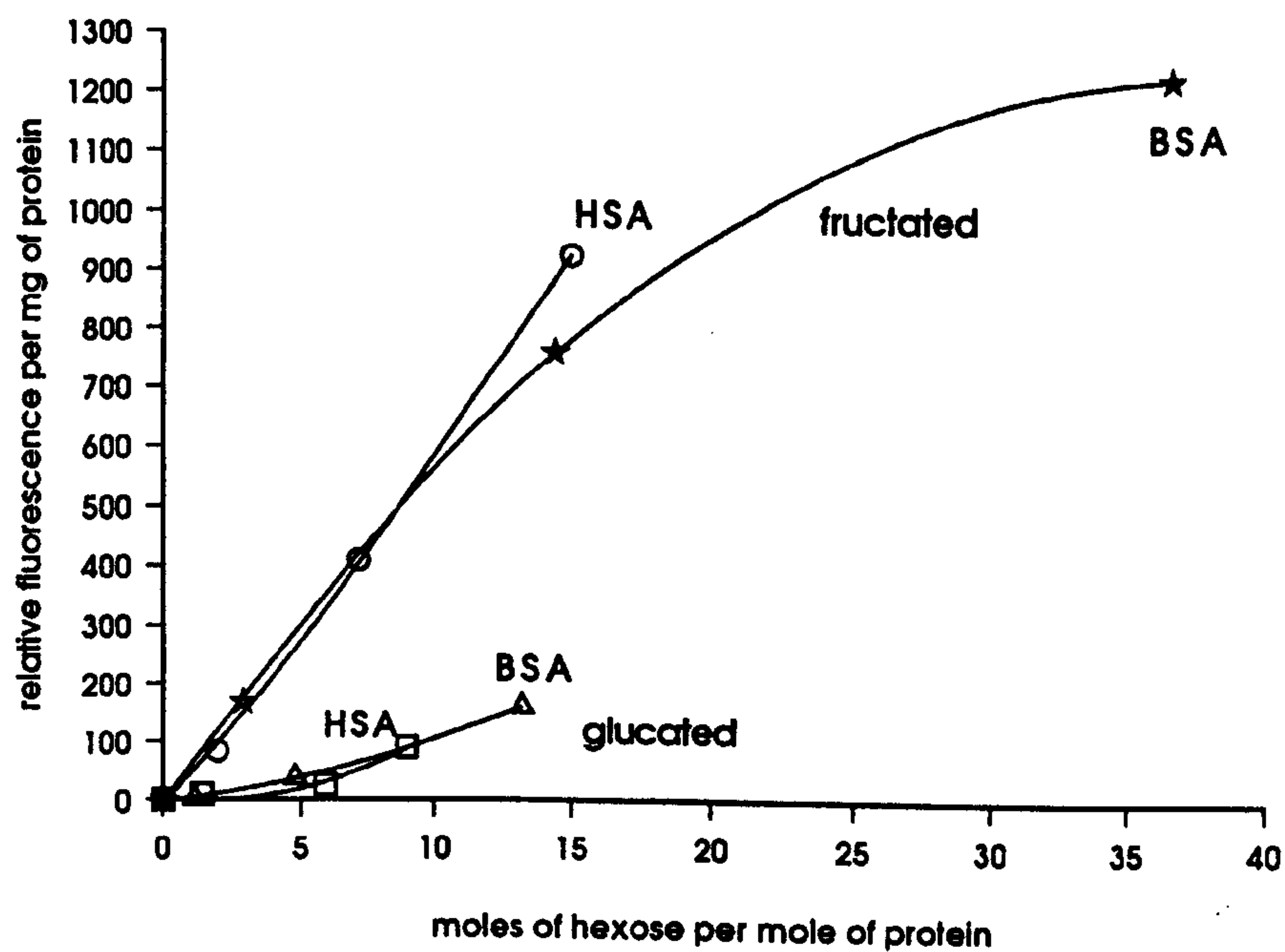


Fig. 3.4.1.5. Comparison of development of fluorescence, (excitation at 350nm and emission at 420nm) due to glycation, with the total incorporation of hexose into HSA, BSA and RNase. Proteins were glycated with either fructose or glucose (0.1M) for 7 days.



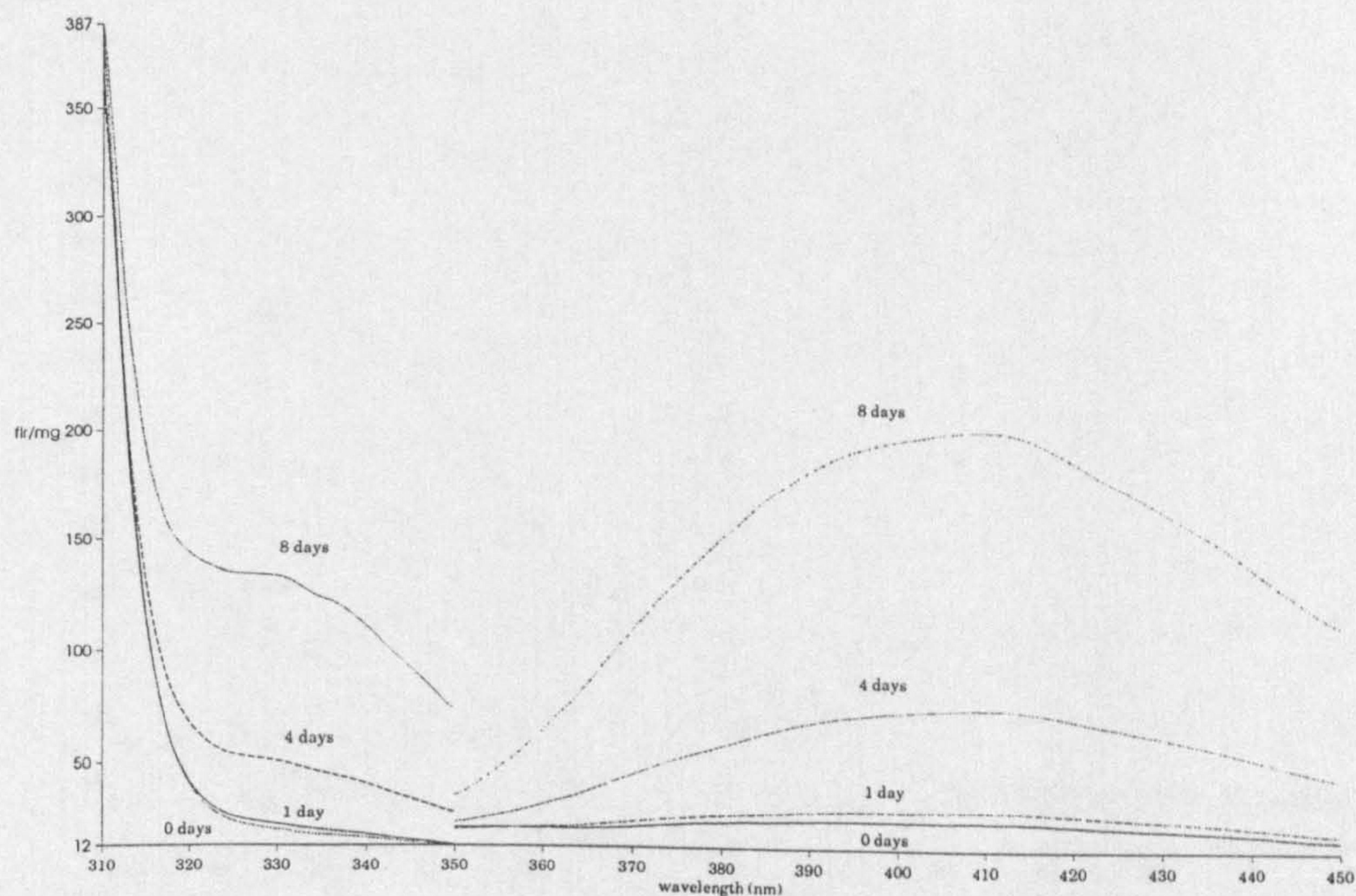
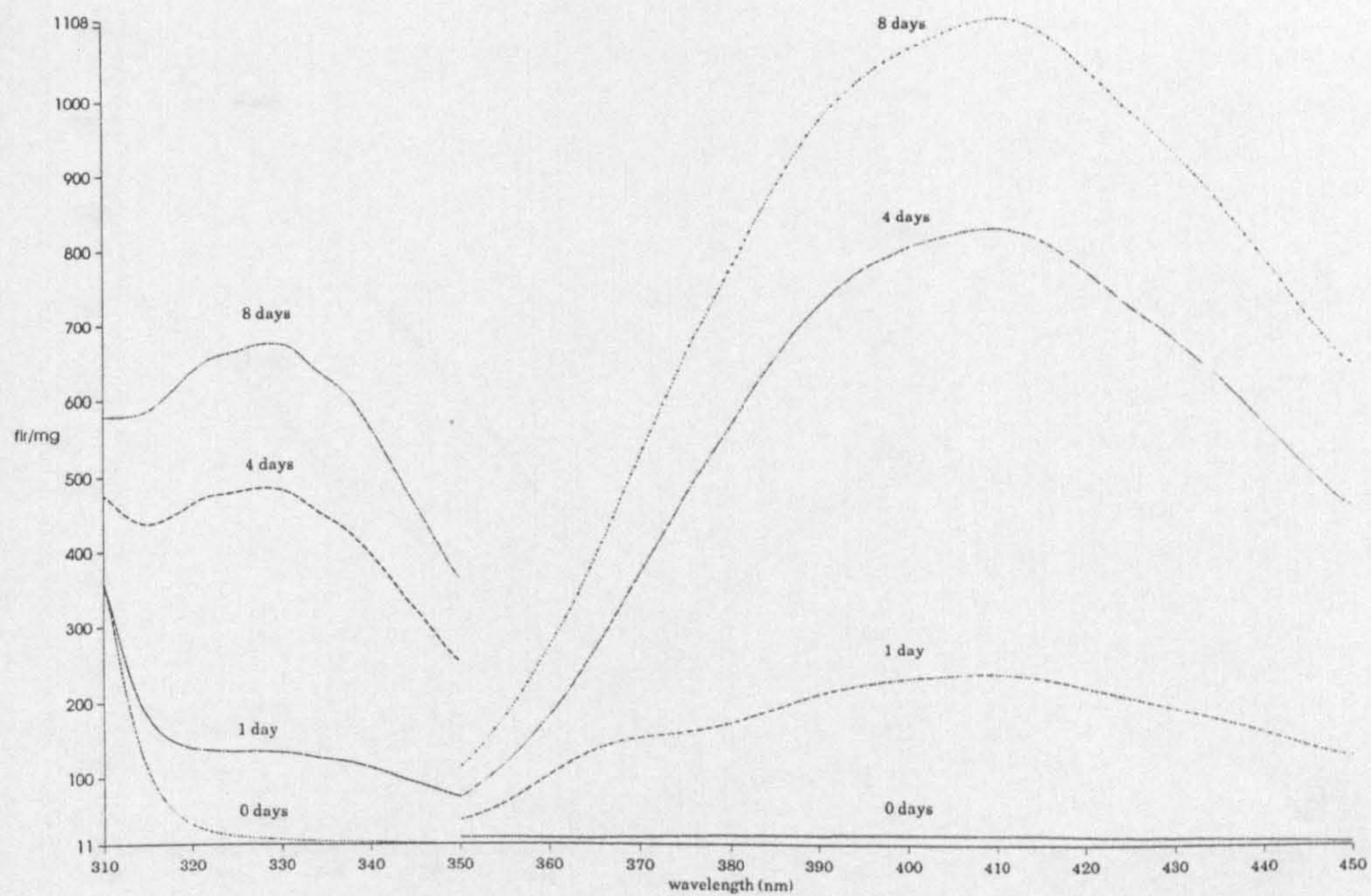


Fig. 3.4.1.6. Fluorescence spectra at pentosidine wavelengths of glycated BSA. The upper part of the figure illustrates the fluorescence spectra of BSA fructated for a range of time, up to 8 days. The lower part of the figure illustrates the same fluorescence spectra when the glycation sugar is glucose.



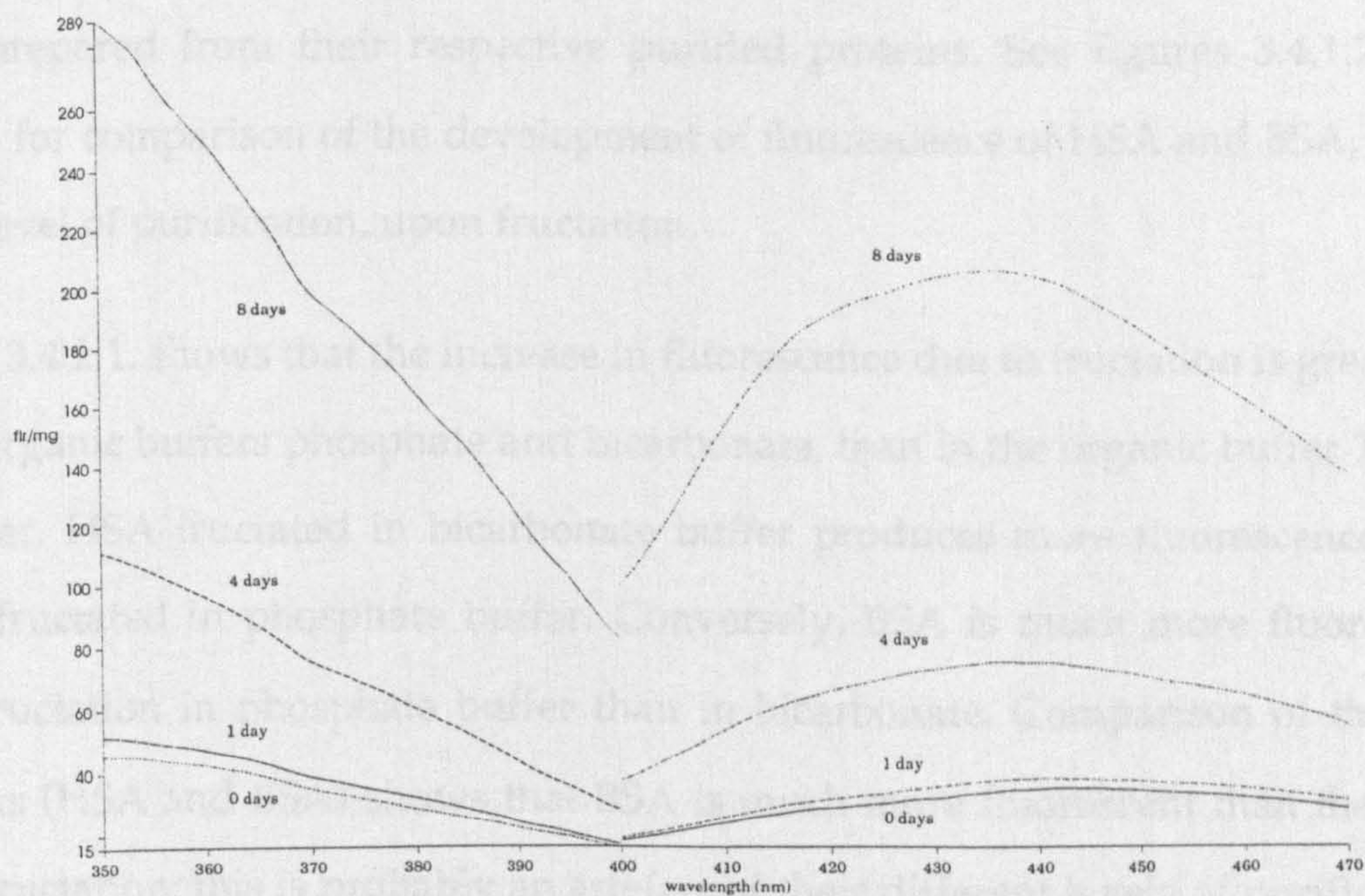
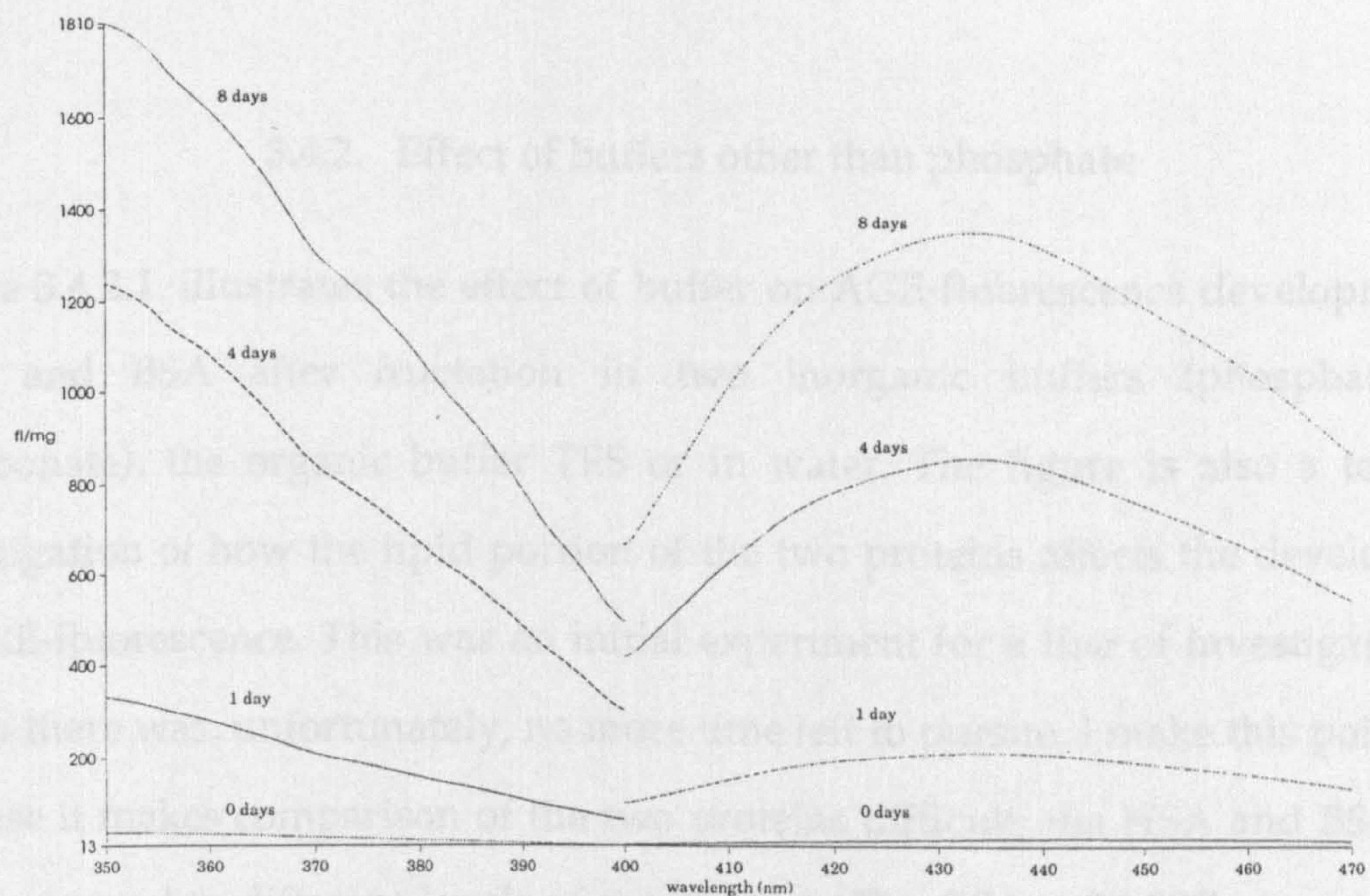


Fig. 3.4.1.7. Fluorescence of glycated BSA, at ex. 350nm and em. 420nm. The upper part of the figure illustrates the fluorescence spectra of BSA fructated for a range of time, up to 8 days. The lower part of the figure illustrates the same fluorescence spectra when the glyating sugar is glucose.



### 3.4.2. Effect of buffers other than phosphate

Figure 3.4.2.1. illustrates the effect of buffer on AGE-fluorescence development in HSA and BSA after fructation in two inorganic buffers (phosphate and bicarbonate), the organic buffer TES or in water. The figure is also a tentative investigation of how the lipid portion of the two proteins affects the development of AGE-fluorescence. This was an initial experiment for a line of investigation for which there was, unfortunately, no more time left to pursue. I make this point here because it makes comparison of the two proteins difficult; the HSA and BSA have been prepared to different levels of purification. The BSA is 96-99% pure whereas the HSA is essentially globulin free (>99% pure) and the delipidated forms have been prepared from their respective purified proteins. See figures 3.4.1.2. and 3.4.1.3. for comparison of the development of fluorescence of HSA and BSA, of the same level of purification, upon fructation.

Figure 3.4.2.1. shows that the increase in fluorescence due to fructation is greater in the inorganic buffers phosphate and bicarbonate, than in the organic buffer TES or in water. HSA fructated in bicarbonate buffer produces more fluorescence than when fructated in phosphate buffer. Conversely, BSA is much more fluorescent after fructation in phosphate buffer than in bicarbonate. Comparison of the two proteins (HSA and BSA) shows that BSA is much more fluorescent than the HSA upon fructation; this is probably an artefact of their different levels of purification; compare this result with those presented in figures 3.4.1.2. and 3.4.1.3.

Figure 3.4.2.1. illustrates that fructation of delipidated HSA and BSA produces different fluorescence than the native forms of the protein. Delipidated BSA develops less fluorescence than its native form, which is consistent with the results of Cochrane (1995) [124]. Delipidated HSA, however, develops more fluorescence



than its native form. If time had been available I would like to have checked this result using a lower level of purification of the HSA, this is because the number of steps involved in the purification of the delipidated HSA (used for Fig. 3.4.2.1.) may have denatured the protein. Denaturation of HSA would allow glycation of lysine residues otherwise unavailable in the non-denatured molecule; thus the extra Amadori or Heyns products formed would produce more AGEs (including fluorescence).

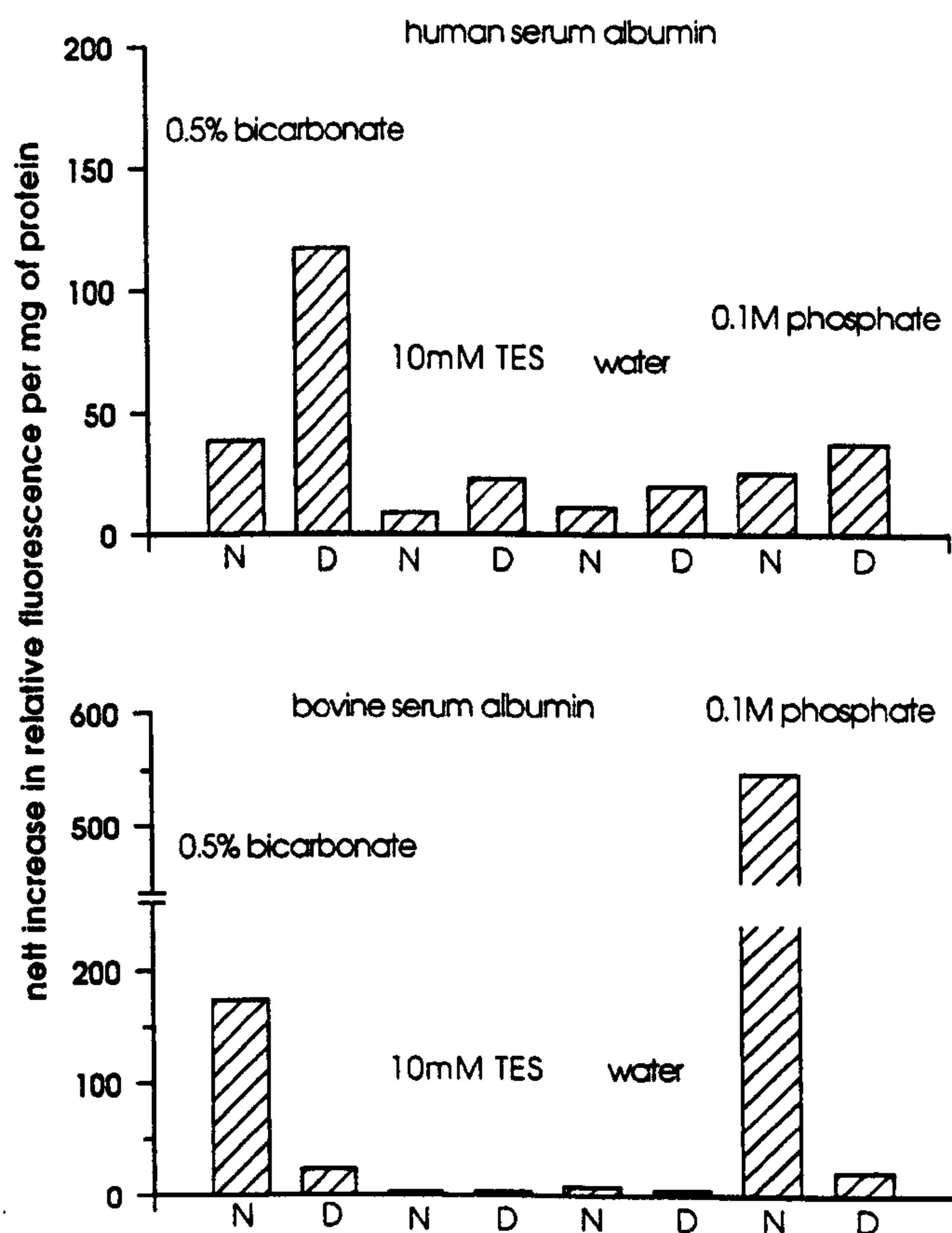


Fig. 3.4.2.1. The effect of using different buffers on development of pentosidine-linked fluorescence of HSA and BSA. The N and D labels indicate the native and delipidated form of that protein. The buffers are indicated in pairs of columns on the histograms. The results are expressed as the net increase in fluorescence after 7 days glycation with 0.1M fructose.

### 3.4.3. Relationship between sugar concentration and AGE formation

Figure 3.4.3.1. illustrates the relationship between the glycating sugar concentration and development of pentosidine-linked fluorescence. The fluorescence prior to glucation or fructation has been subtracted from the results such that the net change due to glycation is apparent. The figure reveals that, for this concentration of BSA ( $10\text{mg ml}^{-1}$ ), increasing the concentration of fructose above  $0.1\text{M}$ , does not produce any additional AGE-fluorescence. At  $0.1\text{M}$  fructose and  $10\text{mg ml}^{-1}$  BSA the sugar to protein molar ratio is 667:1.

The development of fluorescence of BSA as a result of glucation does not plateau off to a similar degree as fructation during the time investigated by this experiment. There is a rate change in the development of fluorescence between  $0.2$  and  $0.4\text{M}$  glucose, which could be the change in rate analogous to that of  $0.1\text{M}$  fructose. It would have been useful to glucate for longer times to confirm this hypothesis.



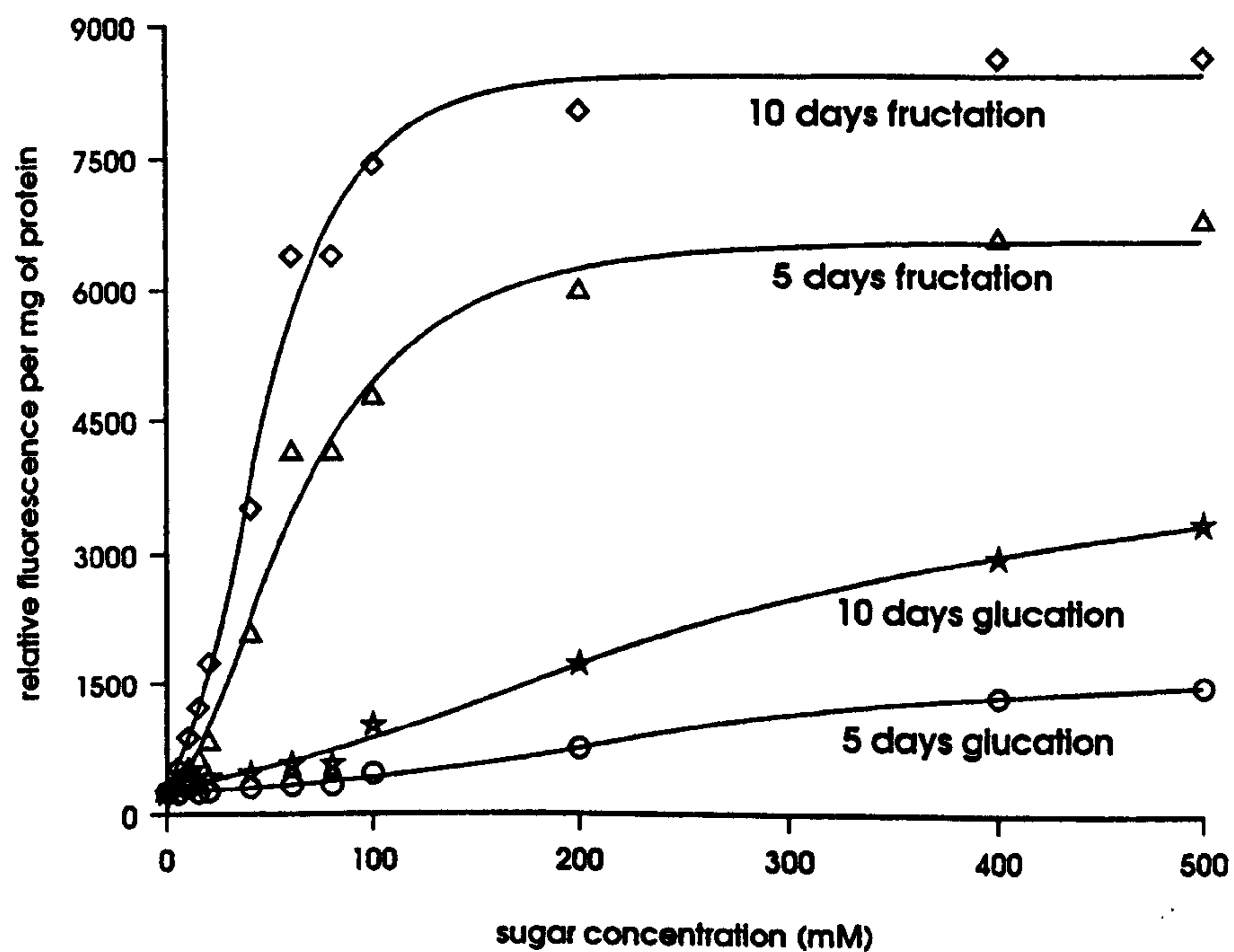


Fig. 3.4.3.1. Relationship between pentosidine-linked fluorescence, produced after 5 and 10 days fructation or glucation, and sugar concentration. The figures are adjusted to take into account the fluorescence prior to glycation.

#### 3.4.4. Formation of AGE-chromophores

Maillard browning is very important to the food industry, it occurs only after all other types of AGE have begun to form and is responsible for colour, aroma, flavour and, with other AGEs, texture of cooked foods [173 and 212]. A spin off from the food industry are the preparations used to artificially tan people; dihydroxyacetone in the tanning preparations reacts with Heyns product (aldehyde rather than the ketone of Amadori product) to form pyrroles that absorb in the blue end of the visible spectrum [174].

Development of AGE chromophores, as in Maillard browning, can cause problems for the study of glycation. Figure 3.4.4.1. illustrates an increase in absorbance of native BSA, at 280nm, due to glycation with 0.2M glucose for up to 20 days. Taken at face value it would indicate that the protein concentration increases as a result of glycation. The same samples, however, when assayed for protein concentration using the Pierce protein assay (see page 61 for a description of the assay), showed that there was no significant increase in protein concentration as a result of glycation (results not shown). Therefore these results indicate that there is production of chromophores that absorb at 280nm as a result of glucation.

Figure 3.4.4.2. shows the absorbance spectra of native HSA at several time points during fructation (0.1M fructose); the lowest line represents the absorbance before glycation and the upper-most line after 8 days fructation. The increasing absorbance, between 250 and 350nm, that develops during fructation represents the production of AGE chromophores that absorb in the UV range.

The left hand side of figure 3.4.4.3. shows the absorbance spectra of four samples containing  $\beta$ -lactoglobulin and fructated lysozyme in bicarbonate buffer; the free fructose used to glycate the lysozyme has been dialysed away. Sample (a) contains

lysozyme, that was 'minimally fructated' (12 hours at 4°C in bicarbonate buffer using 0.005M fructose), to which has been added native  $\beta$ -lactoglobulin. Sample (b) is similar except that the lysozyme has additionally been fructated for 24 hours at 37°C in phosphate buffer using 0.5M fructose. Samples (c) and (d) are samples (a) and (b) respectively after 7 days sugar-free re-incubation.

The right hand side of figure 3.4.4.3. shows the net fluorescence of the two types of samples after the 7 days of sugar-free re-incubation, i.e. fluorescence of sample (c) minus that of (a) and similar for (d) and (b). The range of samples containing lysozyme that had been fructated for periods of time intervening the samples shown, exhibited spectra in between those illustrated in figure 3.4.4.3. and have been omitted for clarity.

The absorbance in figure 3.4.4.3. is expressed per mg of protein to enable comparison of proteins that were diluted to different extents. The samples were diluted to different extents to bring the absolute absorbance below 1.5 and thus within the bounds of the Beer-Lambert law. The absorbance of non-glycated lysozyme with  $\beta$ -lactoglobulin was unchanged by sugar-free re-incubation.

The right hand side of figure 3.4.4.3. shows that the net absorbance of both types of sample increased at 220nm as a result of sugar-free re-incubation. It is the peptide bond of proteins that absorbs in this region, and shifts in the position of the  $\lambda_{\text{max}}$ , up or down the spectra, usually indicate a change in the type of secondary structure. An increase in absorbance usually represents a loss of secondary structure [201]. Thus the right hand side of figure 3.4.4.3. indicates that sugar-free re-incubation of fructated lysozyme with native  $\beta$ -lactoglobulin, results in a loss of secondary structure that is proportional to the time for which the lysozyme was fructated.



The tyrosine and tryptophan residues of a protein absorb at 275-300nm. The absorbance of these residues decreases slightly if they become more exposed to an aqueous solvent, they are therefore sensitive indicators of denaturation. The absorbance of the sample containing 'minimally fructated' lysozyme decreases slightly in this region upon 7 days sugar-free re-incubation which suggests some degree of denaturation. Any change in the absorbance, due to sugar-free re-incubation, of tyrosine and tryptophan residues of the sample containing the more extensively fructated lysozyme, is masked by the development of AGE-chromophores.

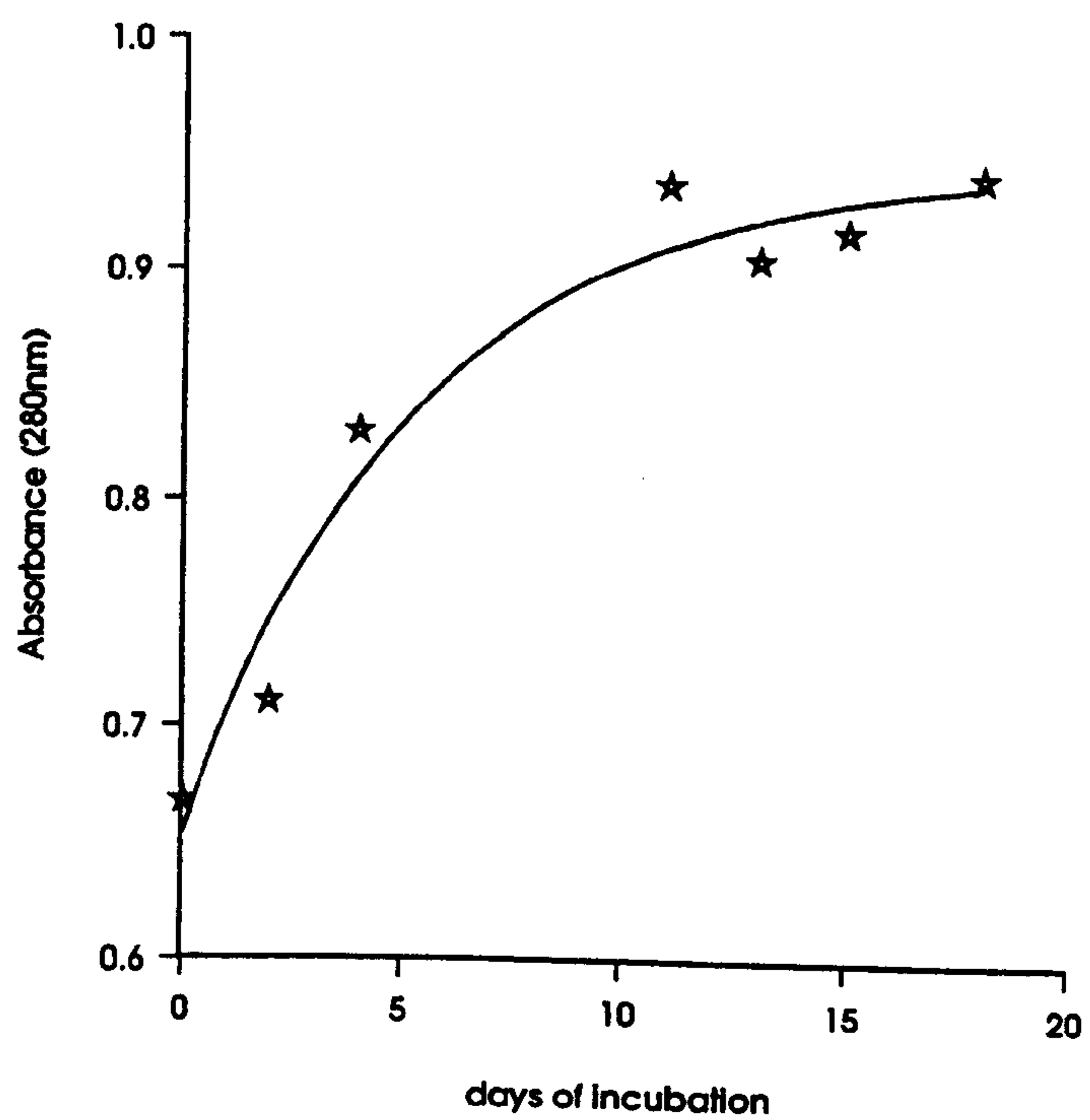


Fig. 3.4.4.1. Increase in absorbance at 280nm of BSA glycated (0.2M glucose) for up to 20 days.

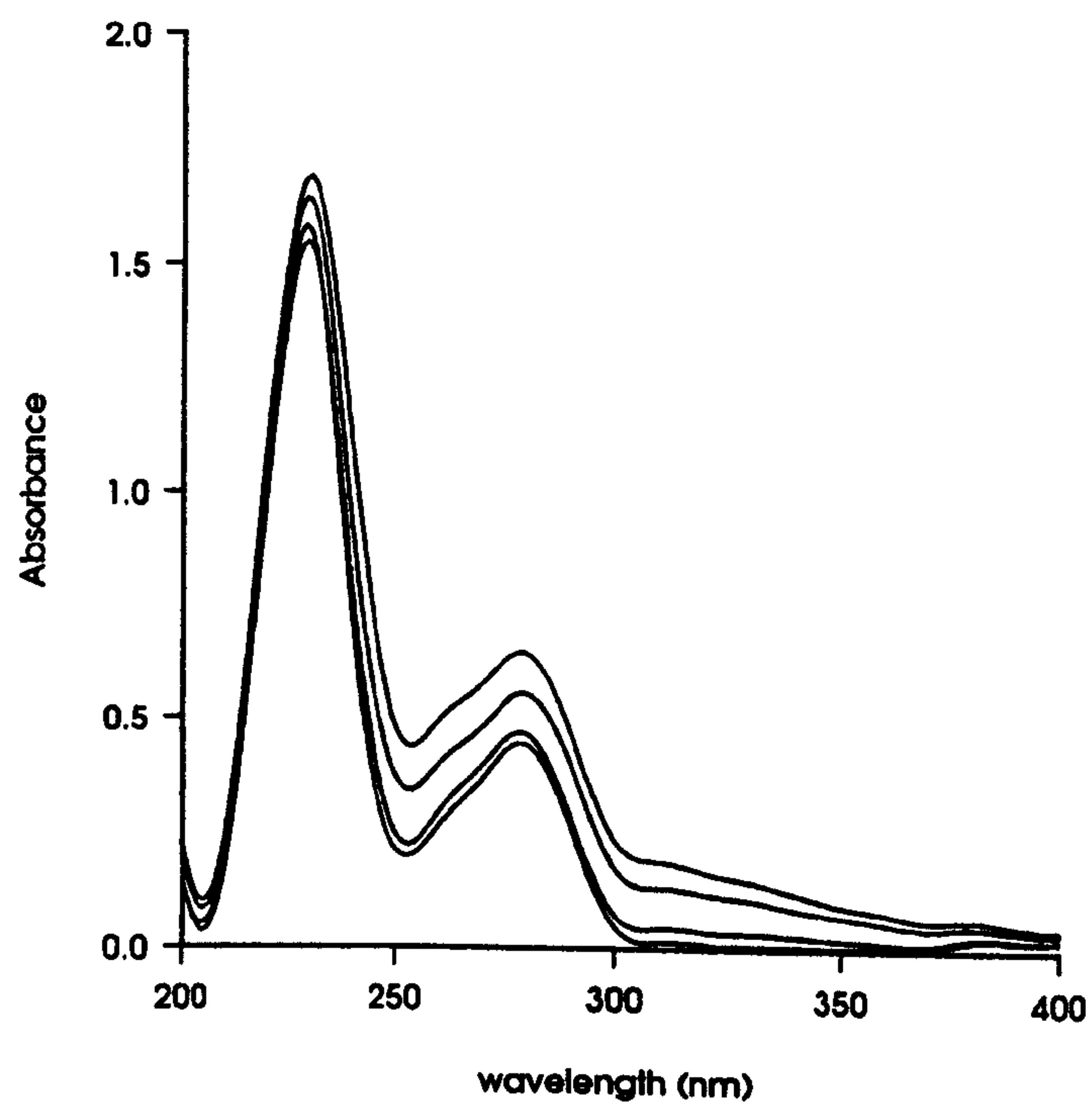


Fig. 3.4.4.2. Absorbance spectra of native HSA (lowest line) and after 2, 4 and 8 (upper line) days fructation (0.1M fructose) respectively.



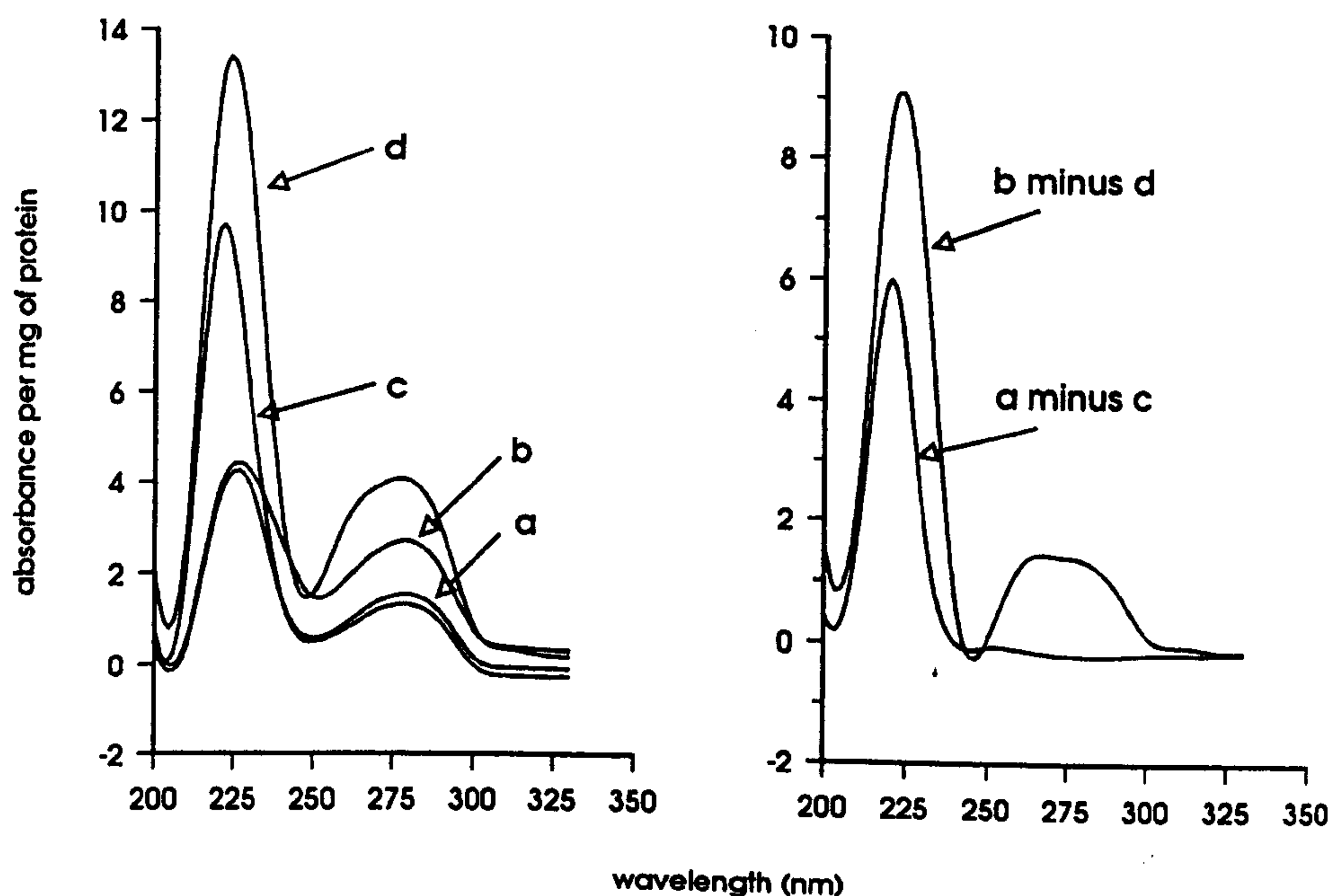


Fig. 3.4.4.3. Left: Absorbance spectra of fructated lysozyme before and after sugar-free re-incubation. The notations represent; a and c = minimal fructation before and after re-incubation; b and d are similar samples but the lysozyme had additionally been fructated for 24 hours. See the text for a fuller explanation of the conditions for fructation. Right: a difference absorption spectra of the left graph, i.e. the change in absorbance due to sugar-free re-incubation; the lower spectra contained minimally fructated lysozyme while the upper had additionally been fructated for 24 hours.

### 3.5. Discussion

#### 3.5.1. Incorporation of hexose

The incorporation of glucose and fructose into BSA, HSA and RNase is illustrated in the graphs of figure 3.4.1.1. Direct comparison with other published work is difficult because of the different glycation conditions used, however the results of some articles are summarised in the table below;

days of glucation	glucose conc.	protein	protein concentration	moles of glucose per mole of protein	ref.
10	0.5M	BSA	6mg ml <sup>-1</sup> (0.09mM)	10	[14]
7	0.02M	RSA	7mg ml <sup>-1</sup> (0.105mM)	1	[141]
1	0.005M	HSA	40mg ml <sup>-1</sup> (0.6mM)	0.5	[143]
7	0.5M	HSA	20mg ml <sup>-1</sup> (0.3mM)	12	[88]

The results for incorporation of glucose into HSA or BSA presented in this thesis are similar to those reported by McPherson *et al* (1988) and Suarez *et al* (1989) [88 and 14]. The differences in the exact figures are likely to be due to differences in glucose and protein concentrations. Figure 3.4.1.1. clearly shows, for all three proteins, that fructose incorporates at a faster rate than glucose of equal concentration. It is unwise to make any conclusions from the results of hexose incorporation into RNase because of the poor protein recovery (10%) from the wash procedure used to remove unbound radiolabelled monosaccharide. McPherson *et al* (1988) reported that glucose was incorporated 8 times faster into HSA than the same concentration of fructose and at similar rates into RNase [88].

The results presented here are in complete contrast to the results of McPherson *et al* (1988) in the case of HSA, but similar to those presented for RNase.

Cox (1991) stated that out of the 59 lysine residues present in serum albumin, ten were known to be readily glycated [121]. Figure 3.4.1.1. and the literature quoted in the table above, indicate that while HSA and BSA will incorporate approximately this amount of glucose under these conditions, fructose is incorporated to a much greater extent. Up to 37 moles of fructose per mole of BSA were recorded in the experiment used in this thesis, there are several possible reasons for this result.

The first reason could be experimental error; if time had been available I would have liked to repeat the experiment on a number of occasions and compare the results statistically. It is possible that the washing procedure used to remove non-covalently bound hexose was not vigorous enough, thus producing an overestimation of the hexose incorporated. Alternatively the calculation of the number of incorporated hexose residues, from the counts of the stock sugar, was inaccurate; if I was to repeat the experiment I would use a range of standards containing a fixed concentration of protein and monosaccharide but with varying proportions of the labelled hexose.

Another reason for the high number of moles of fructose incorporated into BSA could be the involvement of amino acid residues other than the 10 lysines known to become glycated, or more of the 59 lysine residues are involved than the 10 that are initially glycated [121]. This could include the other lysine residues, which may become more readily fructated as the conformation of the BSA is altered during glycation. Alternatively other amino acid residues react with fructose directly or with a post-Heyns compound and thus incorporate radiolabel; arginine residues have been reported to take part in fructation [117]. The involvement of



amino acids, other than lysine, in fructation could be confirmed by radiolabelling coupled to amino acid analysis (if the fructose-protein adduct is acid stable), this could be used to distinguish between incorporation on to lysine or arginine residues.

A third possible reason for a high level of incorporation of fructose in relation to lysine residues of serum albumin, involves the reaction of fructose with the secondary amino group of Heyns product as proposed by Suarez *et al* (1991) [87]. Such a reaction would produce bispyrroles involving two residues of fructose and one lysine residue. Alternatively, free fructose could combine with the fructose residue of Heyns product to form a cyclic acetal; interestingly the formation of cyclic acetals and rearrangement of Heyns product are both acid catalysed. Either of these two explanations would allow incorporation of more than 10 moles of fructose per mole of serum albumin; but only the latter can explain the incorporation of more than 20 moles of fructose onto 10 lysine residues (by perpetuation of acetal formation).

A faster rate of incorporation of fructose than glucose would explain the higher rate of development of AGE-fluorescence (see figures 3.4.1.2. and 3.4.1.3.). A higher rate of development of fluorescence upon fructation, compared to glucation, confirms similar reports for BSA, RNase, Hb and collagen [14, 113, 88, 83, 198 and 199].

Direct comparison of AGE production with incorporation are shown in Figs. 3.4.1.4. and 3.4.1.5. The figures illustrate the relationship between glycation and development of fluorescence. When the *in vivo* produced fluorescence of the proteins has been taken into account there is little difference between HSA and BSA; both proteins upon glycation exhibit the same amount of fluorescence per mole of incorporated monosaccharide. It is only because BSA incorporates glucose

and fructose more quickly than HSA that it develops a higher fluorescence than HSA in the same time period. Thus the results indicate that for up to 15 moles of fructose or 9 moles of glucose per mole of protein, BSA and HSA are developing very similar, if not identical, AGEs. The figures 3.4.1.4. and 3.4.1.5. also show that after incorporation of 15 moles of fructose per mole of BSA, the rate of AGE-fluorophore formation is not at the same rate as incorporation of fructose, even though figure 3.4.1.1. shows unchanged incorporation. Such halting of development of fluorescence could mean that the fluorophores or their precursors are substrates for an AGE formed at a later stage in the Maillard reaction.

The upturned curves of glucated albumin in figures 3.4.1.4. and 3.4.1.5. suggest a lag between incorporation of glucose and development of fluorescence; i.e. post-Amadori reactions are slower than glucation and the rate of incorporation of glucose, as Amadori product, is only slowed when equilibrium between Schiff's base and Amadori product is reached. An alternative hypothesis that could also explain the results is that a certain amount of glucose needs to be incorporated before AGE-fluorescence will develop; i.e. a fluorophore is made by the reaction of two or more precursors both of which need to be present for it to form. The two hypotheses could be tested by glucating for a short time (i.e. less than 2 days using 0.1M glucose), removing the excess free sugar and re-incubating. If the first hypothesis is true then fluorescence will develop during sugar-free re-incubation; if no fluorescence develops then it could mean that the latter hypothesis is true because there is insufficient precursor present, on the protein, for a fluorophore to form.

Figures 3.4.1.2., 3.4.1.3., 3.4.1.6. and 3.4.1.7. all indicate that fluorescence develops faster upon fructation than glucation. The latter two figures are fluorescence spectra that I have used to observe development of AGE formation. Comparison of



the shape of the spectra produced either by glucation or fructation, does not, unfortunately, reveal any difference in the fluorophores produced by the two monosaccharides; except fructation produces fluorescence of a higher intensity than glucation. Figures 3.4.1.6. and 3.4.1.7. illustrate the fluorescence spectra used to measure AGE formation throughout this thesis, i.e. the pentosidine wavelengths and those specific for the  $\lambda_{\text{max}}$  of glycated albumin. These wavelengths were selected to enable comparison of the results of different experiments used to prepare this thesis and to other literature.

### 3.5.2. Effect of buffers other than phosphate

The effect of buffers on AGE formation is quite profound. Baynes (1989) reported that incorporation of glucose did not occur to the same extent in the organic buffer TAPSO, than in the anionic inorganic buffers such as phosphate, arsenate and bicarbonate [140]. The same group have reported similar results for the organic buffer MOPS and in chapter 5. similar results are presented for HEPES buffer [138]. The results presented in figure 3.4.2.1. confirm the observations of the Baynes group; i.e. fluorescence development from glycation only occurs in inorganic buffers.

It is the free metal contamination of the inorganic buffers that is thought to take part in AGE formation. This is because chelation of the free metal, in the inorganic buffers or inclusion of free radical scavengers during glycation, inhibits AGE formation (see chapter 4. for discussion) [179, 205]. The results of figure 3.4.2.1., however, show that it is not just the free metal of the inorganic buffers that affects AGE formation. At the concentrations and pH used, bicarbonate buffer potentially contains more free metal (maximum concentrations of 16 $\mu$ M copper and 9 $\mu$ M iron) than phosphate buffer (maximum concentrations of 5 $\mu$ M copper and 6 $\mu$ M iron); yet BSA when fructated for 7 days in phosphate buffer, exhibits three times more



fluorescence than if the buffer were bicarbonate. If I assume that the minimum concentration of free metal quoted by the manufacturers is similar to the actual concentration of those ions, then some other component of the buffers must also be taking part in the reaction to explain the observed results. A definitive assay is needed for the free metal content of the buffers, to confirm or disprove this hypothesis. Anions of the buffers are candidate compounds that could take part in AGE formation. Watkins *et al* (1985) proposed that inorganic phosphate bound in the active site of RNase was catalysing Amadori rearrangement on lysine residues nearby. It is possible that phosphate forms a ligand with BSA near lysine residues that take part in glycation and catalyses rearrangement, in this case to Heyns product. Conversely native HSA develops more fluorescence in bicarbonate buffer than phosphate, which is consistent with the free metal content of the buffers, but the difference is not large. The differences in the results for HSA and BSA shown in figure 3.4.2.1. are likely to be due to their different levels of purification; although, generally, BSA does develop slightly more intense fluorescence than HSA (see figures 3.4.1.2. and 3.4.1.3.).

Delipidation of BSA inhibits development of fluorescence upon glycation (Fig. 3.4.2.1.); indicating that the lipid component of BSA takes part in AGE formation. Delipidated HSA, however, develops a more intense fluorescence than its native counterpart, this is probably an artefact of the number of steps used to purify this protein. Denaturation of the HSA would lead to a higher rate of glycation and thus AGE-formation due to exposure of more lysine residues. If, however, the result is not due to denaturation of delipidated HSA, it would indicate that the lipid component of HSA has a protective role against development of AGEs. The lipid component of serum albumin is very important in AGE formation because it will also undergo peroxidation. Sugar derived AGEs and products of lipid

peroxidation have very similar properties; additionally the presence of one type has been reported to enhance the production of the other [163 and 164].

### 3.5.3. Relationship between sugar concentration and AGE formation

To obtain large amounts of AGEs, some studies have used large non-physiological sugar concentrations with very long incubation times, e.g. 90 days incubation of various proteins with 0.5M glucose, to produce AGE-proteins against which antibodies were raised [145 and 146]. I am critical of the physiological relevance of AGEs produced after such harsh glycation and thus the antibodies raised against them. Figure 3.4.3.1. shows the development of AGE-fluorescence of BSA due to 5 and 10 days glycation at various concentrations of glucose and fructose. The figure illustrates that increasing the concentration of fructose between 0.1M and 0.5M does not result in a corresponding increase in AGE-fluorescence; whereas at less than 0.1M fructose the increase in fluorescence is directly proportional to the concentration. The relationship between glucose concentration and development of fluorescence is not so straightforward as for fructose, which is probably a reflection of the lower reaction rate of glucation compared to fructation. If the experiment were carried on for a longer time of glucation, I would have expected to see similar results to those produced with fructose because there is a change in the slope of the graphs between 0.2 and 0.4M, which could be the beginnings of a plateau.

### 3.5.4. Formation of AGE-chromophores

HSA and BSA have been reported to have an increased absorbance, around 290nm, which is proportional to the period of glycation [147 and 14]. Figures 3.4.4.1. and 3.4.4.2. show the increase in absorbance at 280nm caused by glucation of BSA and the absorbance spectra of HSA fructated for various time periods up to



8 days. The apparent increase in absorbance at 280nm would suggest an increase in protein concentration as a function of the time of glucation; however, the same samples assayed for protein using the Pierce protein method (page 58) indicated no such change in protein concentration. The increase in absorbance at 220 and 290nm seen in figure 3.4.4.2. could be due to changes in protein conformation since these wavelengths correspond to absorbance by the peptide bond and the amino acids tryptophan and tyrosine respectively. A change in conformation would alter the absorbance at 220 and 280nm but it would produce peaks with narrow ranges of wavelengths. Figure 3.4.4.2. however, shows increasing absorbance from 250 to 350nm, as a function of the time of fructation, which can only be due to formation of AGE-UV-chromophores. The UV-chromophores that absorb in the 250 to 350nm region are probably the excitation wavelengths of AGE-fluorophores. Observation of the increase in absorbance at these wavelengths could be used as a simple method for observing glycation, without having to resort to fluorescence.

The last figure of this chapter (Fig. 3.4.4.3.) illustrates the absorbance of  $\beta$ -lactoglobulin together with one of two extremes of fructated lysozyme, before and after 7 days sugar-free re-incubation. Sugar-free re-incubation of 'minimally fructated' lysozyme (12 hours, 4°C, 0.005M fructose in bicarbonate buffer) with native  $\beta$ -lactoglobulin, produces a large increase in absorbance at 220nm, suggesting a change in the protein secondary structure. This conclusion is supported by the negative absorbance between 270 and 300nm indicating an increased exposure of tryptophan and tyrosine residues to polar solvent [210]. The sample containing the more highly fructated lysozyme (37°C, 0.5M fructose in phosphate buffer) has increased in absorbance at both 220nm and 250 to 300nm. The increase in absorbance at 220nm, again, indicates loss of secondary structure, whereas the broadening of this peak suggests formation of conjugated dienes (which absorb at 230-240nm) on the lipid component of  $\beta$ -lactoglobulin [211]. The



broad increase in absorbance between 250 and 300nm, of the more highly fructated sample, is probably due to production of AGE chromophores rather than changes in protein secondary structure.

Ames (1993) reviewed the work of Kato's group into modification of ovalbumin secondary structure by glucation, and reported that although there was an increase in secondary structure disorder, as measured by circular dichroism. The glycated monomer was more resistant to denaturation than native protein, this was proposed to be due to increased intramolecular cross-links within the glycated monomer [212]. Thus the results of this thesis support the hypothesis that glycation alters protein conformation as well as quaternary structure.

The results of this chapter tell us several things about fructation and AGE production from it. Fructose is incorporated into serum albumin to a greater extent than an equal concentration of glucose over the same time period and this is reflected by the production of AGEs from the respective monosaccharides. Post-Heyns reactions cause changes in the conformation of lysozyme and  $\beta$ -lactoglobulin and also produce AGE-chromophores. Incorporation of fructose into BSA continues after the development of fluorescence halts, indicating the production of non-fluorescent AGEs that are undetected in this experiment. The large number of moles of incorporated fructose per mole of BSA suggests that residues other than lysines are taking part in the Maillard reaction. Lastly AGE formation, from fructose, is influenced by several components of the buffer, such as the free metal content (discussed in chapter 4.) and possibly the buffer anions. The action of the anions is probably by interaction with the protein, catalysing Amadori or Heyns rearrangement.

### 3.6. Summary

1. Fructose is incorporated into protein to a greater extent than glucose and this is reflected in the development of fluorescence.
2. BSA incorporates more moles of hexose and develops a greater fluorescence than HSA; although, for less than 15 moles of incorporated hexose per mole of serum albumin they develop the same fluorescence per mole of incorporated fructose or glucose.
3. Incorporation of fructose into BSA continues after development of fluorescence has ceased.
4. Several components of inorganic buffers take part in AGE formation. One of the components is the free metal that contaminates these buffers (see later chapters); another component is probably the interaction between the protein and the anions.
5. Delipidation inhibits the development of AGE-fluorescence of BSA.
6. If *in vitro* fructation is performed in phosphate buffer at 37°C, for 5 or more days with 0.15mM BSA, maximum fluorescence is developed using 0.1M fructose. Under these conditions higher fructose concentrations do not increase the yield of fluorescence.
7. AGE-chromophores that absorb in the UV-regions (220-370nm) could be used as a simple assay for glycation.
8. Sugar-free incubation of native  $\beta$ -lactoglobulin with 'minimally fructated' lysozyme produces a change in the UV-absorbance of the two proteins that

corresponds to an increase in secondary structure disorder, including the movement of the side groups of tryptophan and tyrosine residues towards a polar environment.



## 4. Protein-bound carbonyl intermediates in the Maillard reaction: colorimetric detection of fructation

### 4.1. Introduction

#### 4.1.1. The need for an assay for fructation

Ahmed and Furth (1992) reported that serum fructosamine, thiobarbituric acid (TBA), periodate and phenylboronate assays all underestimate fructation when compared to fluorescent AGE formation [209]. The fundamental difference in the chemistry of Heyns and Amadori products (Fig. 4.1.1.1.) means that fructation is undetected or underestimated by the chemical assays developed to detect glycation by glucose.

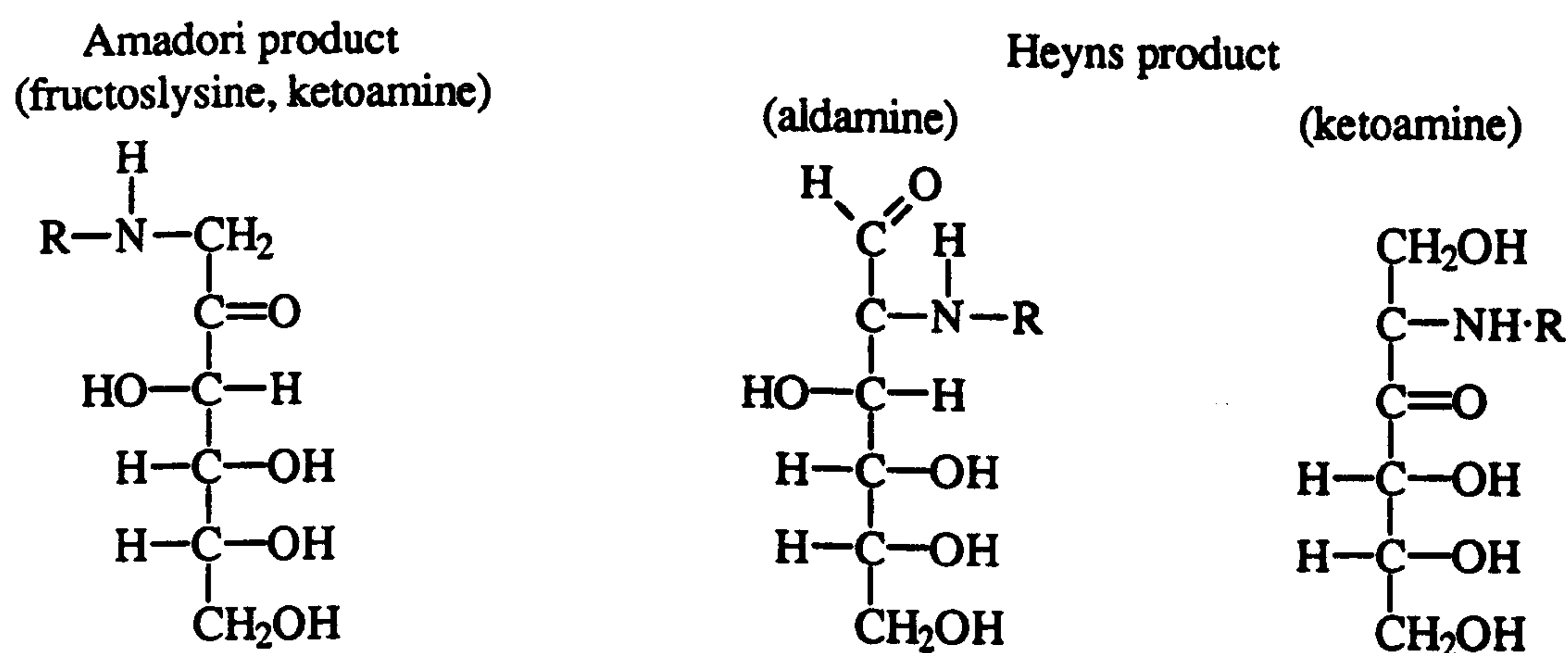


Fig. 4.1.1.1. Amadori and Heyns products.

The implications of fructation *in vivo* were only realised when it was shown to occur using methods that can detect it. One of these methods is measurement of glycated haemoglobin (HbA<sub>1</sub>, see page 21 for more information). The particular fraction of HbA<sub>1</sub> used to assess glycaemic control depends on the assay used, but in poorly controlled diabetics, up to 20% of total Hb has been found in the form of

the HbA<sub>1c</sub> subfraction [148 and 149]. The fractions of HbA<sub>1</sub> other than HbA<sub>1c</sub> are formed by N-terminal glycation of haemoglobin by other sugars such as fructose and galactose. High HbA<sub>1</sub> values have been observed in people who are not diabetic but suffer from hereditary fructose intolerance or galactosaemia [150]. Dietary fructose has also been blamed for the high 'glycosylated haemoglobin' observed in a type 1 diabetic who had good glycaemic control but ate liberal amounts of 'diabetic food' that used fructose as a sweetener. Substitution of the fructose by other forms of carbohydrate brought the glycosylated Hb down to within normal limits with no appreciable change in glycaemic control [151]. Clearly measurement of total glycated haemoglobin can show glycation by other sugars, which could confuse diagnosis of glycaemic control in diabetics, but measurement of HbA<sub>1c</sub> alone misses the contribution of other sugars to glycation of circulating proteins [150].

Evidence of *in vivo* fructation includes fructose derived from the polyol pathway (page 40). Fructose from this source has been implicated in the formation and progression of diabetic secondary complications; e.g. Ten to twenty per cent of the hexose bound to human ocular lens was reported by Walton *et al* (1989) to derive from fructose [111 and 200].

A principle aim of this PhD was to develop a method that would quantify, on a molar basis, protein fructation in a similar manner to the glucation assays.



#### 4.1.2. Reactions of 2,4-dinitrophenylhydrazine

An important method for the detection and identification of saccharides is by reaction with 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones and osazones, see figure 4.1.2.1. Osazones have characteristic absorbance spectra which can allow identification of the parent monosaccharide, although in the case of glucose and fructose they form the same osazone [152 and 153].

DNPH has previously been used to detect glycation. Acharya and Manning (1980 and 1983) used an adaptation of the method described by Fields and Dixon (1971) to detect glycation by glyceraldehyde. These studies were model experiments to show that glyceraldehyde and  $\alpha$ -hydroxyaldehydes could glycate haemoglobin, which in turn prevented aggregation of sickle cell deoxyhaemoglobin. Unreacted DNPH was removed by size exclusion chromatography. The articles showed that protein-bound carbonyl increases with the concentration of aldehyde allowed to glycate a protein over a set period of time [154, 155 and 156]. Fischer and Winterhalter (1981) followed up the work of Acharya *et al* and looked for protein-bound carbonyl groups on glycated haemoglobin isolated from diabetic sera. They reported very little reaction and concluded that the concentration of open chain Amadori product (assumed to be the reactive compound) in haemoglobin was below the resolution of the assay [157]. Ghiggeri *et al* (1986) detected protein-bound carbonyl groups, using DNPH, on glycated albumin purified from diabetic sera. These were attributed to reaction of the DNPH with Amadori product; however, the total amount of carbonyl groups detected were very low in comparison with the total bound carbohydrate [158]. Kobayashi *et al* (1992) have developed a quick method for detection of Amadori product using DNPH; Amadori product is released as 2-keto-glucose under the influence of hydrazine and pressure, this in turn reacts with DNPH to form an osazone which is detected



by its absorbance at 390nm [159]. Ahmed (1992) in his PhD thesis used the DNPH method of Fields and Dixon to detect fructation and it was from here that my investigation started [133 and 156].

The DNPH methods reported by Kobayashi *et al* (1992) and Fields & Dixon (1971) are both limited by insensitivity, this is because there is no separation of excess DNPH from the 2,4-dinitrophenylhydrazone product. The two compounds have overlapping absorbance spectra and thus the excess reagent has a significant absorbance in the same region as that used to detect the product (390nm) [159 and 156]. The sensitivity of assays that do not remove excess reagent can be improved by measuring the ratio of absorbance at 375 and 405nm, and observing the change due to glycation. Absorbance at 375nm corresponds mostly to the DNPH and, as it is in large excess, only decreases slightly due to a small loss of hydrazone. While the absorbance of the 2,4-dinitrophenylhydrazone is shifted higher up the spectrum to 405nm. The DNPH does absorb at 405nm but the increase due to formation of 2,4-dinitrophenylhydrazone is relatively large in comparison to the decrease at 375nm. Thus a measurement of the ratio of 375 and 405nm emphasises 2,4-dinitrophenylhydrazone formation and small changes can be observed. This method, however, when put into practice, by myself, revealed that the increase in sensitivity was not enough to reduce the inter-assay coefficient of variation to an acceptable value (results not shown), so an alternative method that removed excess reagent was sought.

A sensitive method for determining protein oxidation using DNPH was reported by Levine *et al* (1990). It involved the removal of excess DNPH and osazones formed from reaction with free sugar [170]. I have used this method to quantify fructation as a function of carbonyl groups formed on TCA insoluble protein. A

description of the assay protocol and the adaptations I have made to it is covered later in the chapter.

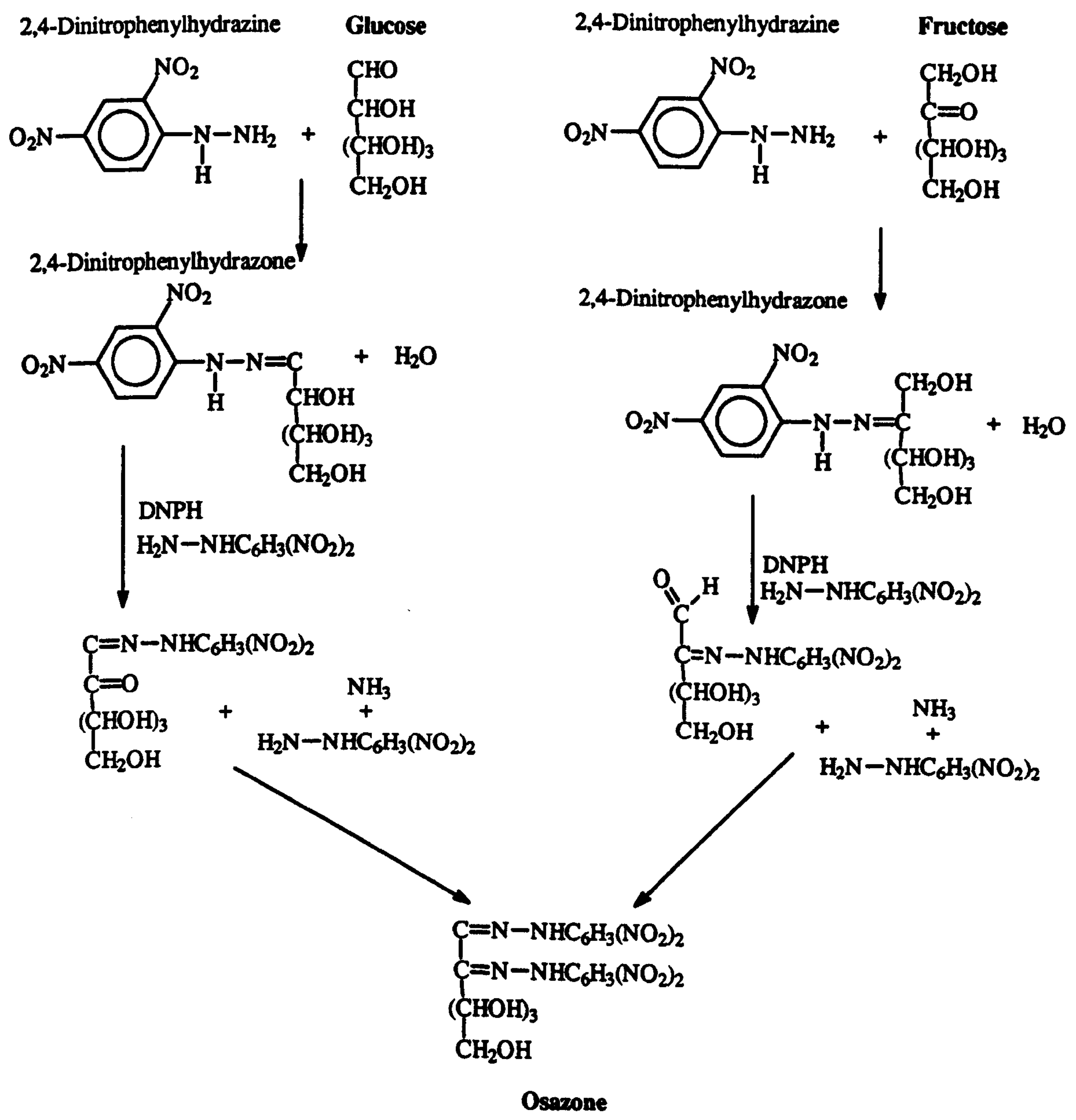


Fig. 4.1.2.1. Reaction of DNPH with glucose and fructose [152].

#### 4.1.3. Role of lipid in the Maillard reaction

This chapter also deals briefly with the effect of lipid on various measures of glycation, this is because lipid oxidation products may also have free carbonyl groups that would react with DNPH. Modification of protein by lipid oxidation products has been reported to produce fluorescence spectra similar to those of AGEs [160, 161 and 162]. The lipid oxidation product malondialdehyde has been reported to accelerate the formation of AGE-chromophores by reaction with Amadori product [163]. Also Bucala *et al* (1993) have reported enhanced production of lipid peroxidation products after formation of AGEs; the group also showed that glycation of low density lipoprotein (LDL) produces AGEs on both the lipid and protein components [164]. Thus the production of lipid peroxides and AGEs is intimately connected, with the formation of one enhancing the production of the other.

#### 4.1.4. Mechanisms of aminoguanidine action

Aminoguanidine and DNPH are both nucleophilic hydrazines and probably have very similar reactive properties [165]. This means that the carbonyl groups detected, upon glycation, by the DNPH assay will also react with aminoguanidine. If in the absence of aminoguanidine these same protein-bound carbonyl groups take part in AGE formation, then their sequestration by aminoguanidine could explain the anti-AGE activity of the hydrazine. Thus investigation of the point in the Maillard reaction detected by DNPH is also an investigation of the inhibitory mechanism of aminoguanidine.

The site of the inhibitory action of aminoguanidine has been investigated by a number of workers and several hypotheses have been proposed. Brownlee *et al* (1986) suggested that aminoguanidine reacts with the carbonyl group of Amadori



product and, by blocking that group, prevents its further reaction, see figure 4.1.4.1.

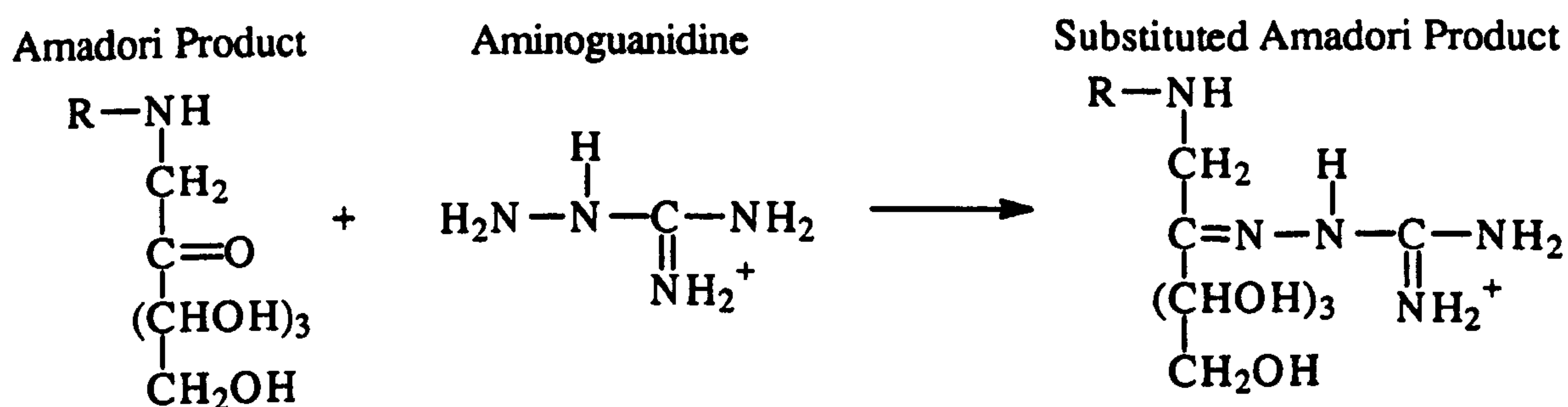


Fig. 4.1.4.1. Putative reaction of aminoguanidine with Amadori product [165].

Blackledge (1993) reported that the yellow chromophore ( $\lambda_{\text{max}}$  313nm) produced when aminoguanidine is used as an inhibitor of glycation, is from its reaction with the glycating sugar [166]. With glucose the chromophore was reported to be probably mostly hydrazone with some osazone, see figure 4.1.2.1.; Blackledge went on to state that labelled aminoguanidine did not react with fructoslysine (Amadori product) and proposed that it reacts with free dicarbonyl intermediates derived from Amadori product, such as 3-deoxyglucosone; this hypothesis is supported by the findings of Edelstein and Brownlee, (1992) [166 and 167]. Requena *et al* (1993) reported that although aminoguanidine would react with Amadori product and block its reactive carbonyl group, this was not a major site of its action. The article also stated that scavenging of glucose by aminoguanidine had little impact on furosine yields, thus the site of action must be elsewhere, again 3-DOG was suggested [168]. Figure 4.1.4.2. illustrates the reaction of aminoguanidine with a range of dicarbonyl compounds proposed by Hirsch and Feather (1994) to be possible intermediates in the Maillard reaction [169]. Since 3-DOG is a free dicarbonyl, its contribution to post-Amadori or post-Heyns reactions could be tested by re-incubating the sugar-free protein solution at 37°C while dialysing continually against new buffer.

R =      a = 3-deoxyglucosone      b = 3-deoxyxylosone      c = glucosone      d = xylosone

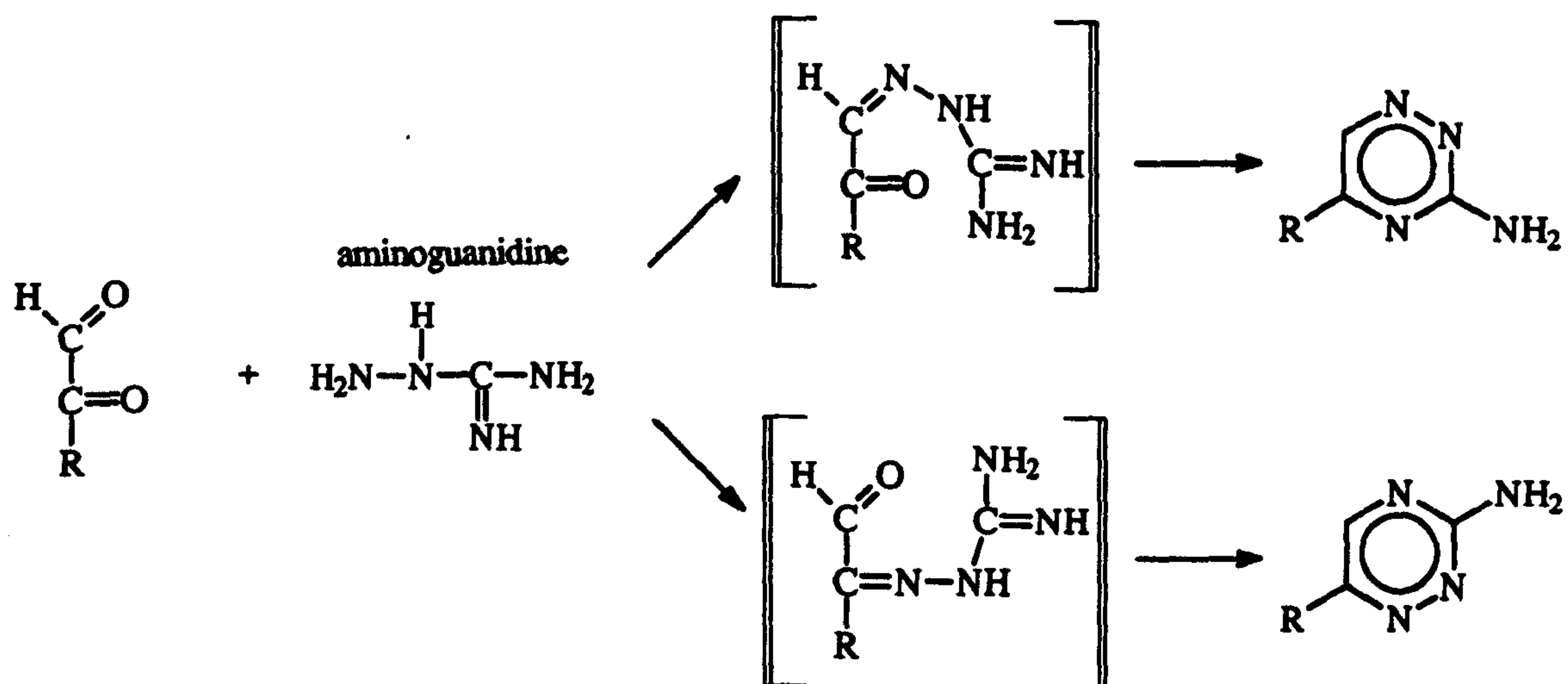
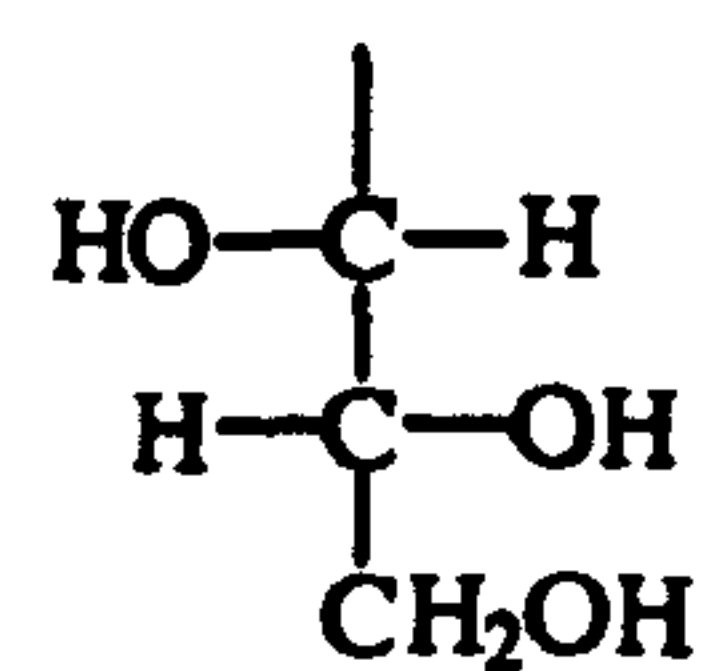
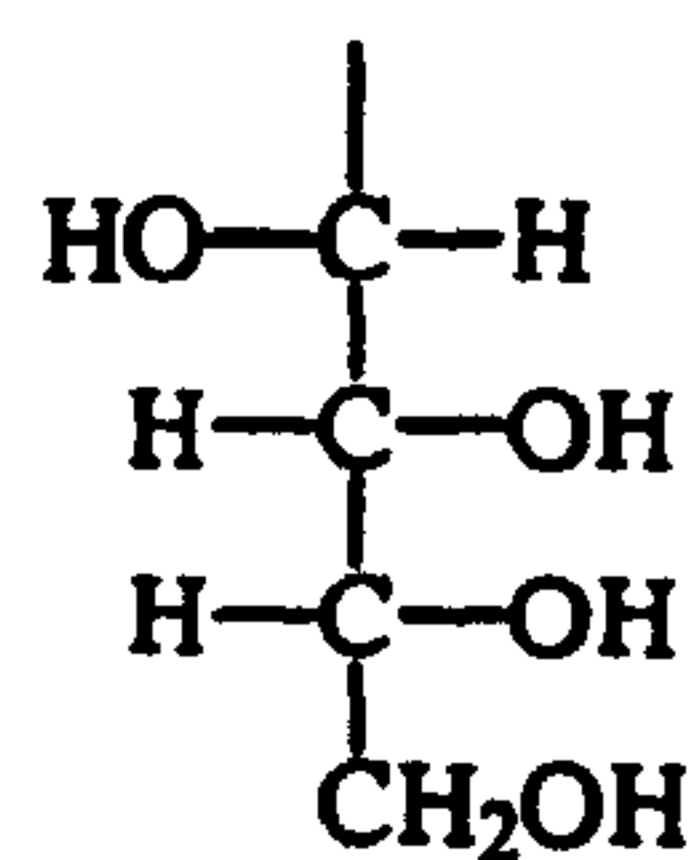
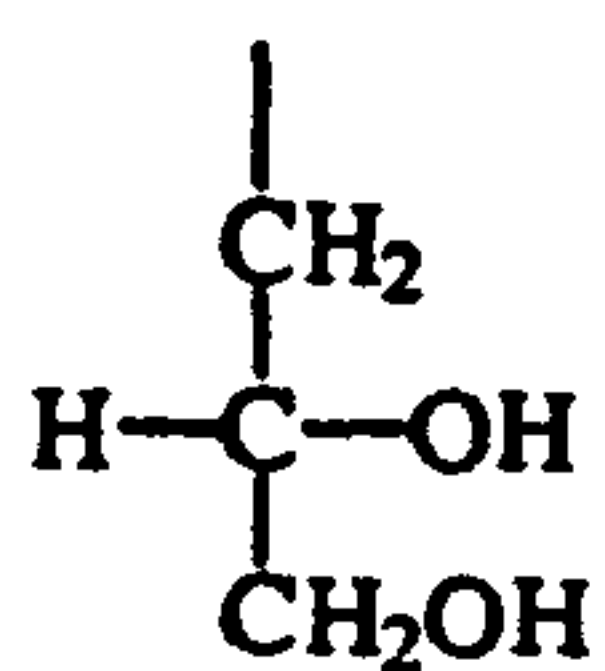
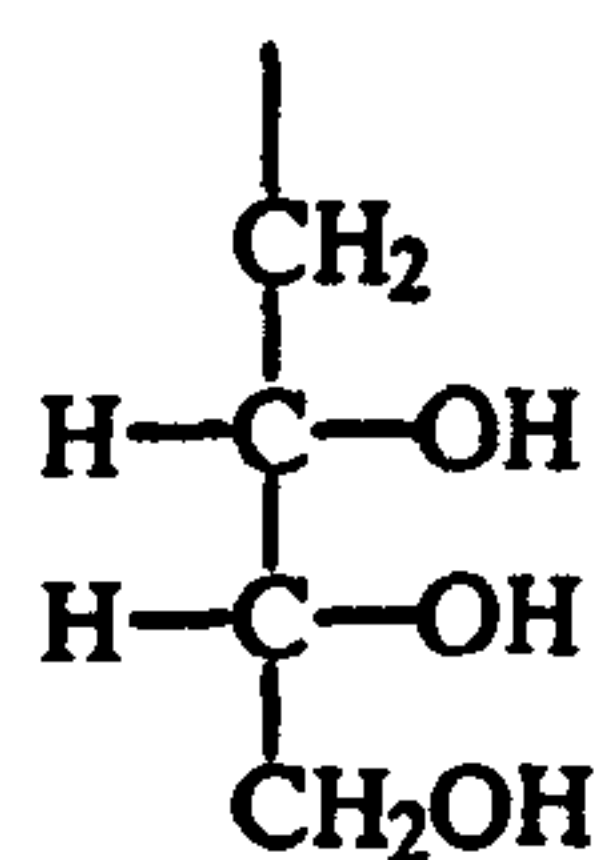


Fig. 4.1.4.2. Reaction of aminoguanidine with some dicarbonyl compounds [reproduced from Fig. 1 of 169].

## **4.2. Aims**

- 1. To develop a quantifiable colorimetric method that would detect glycation by fructose (fructation).**
- 2. To use this method to elucidate the biochemical mechanism of AGE formation from fructose.**
- 3. To investigate the role of lipid in the production of protein-bound carbonyl groups and AGEs.**
- 4. To investigate the site of action of aminoguanidine.**



### **4.3. Methods**

#### **4.3.1. The DNPH assay**

The protocol for detection of glycation using reaction with DNPH is as follows:

1. Place three 100 $\mu$ l aliquots of each sample (containing 1mg of protein) into separate 1.5ml eppendorfs.
2. To two of the 100 $\mu$ l samples, add 400 $\mu$ l of 7mM DNPH made up in 2M HCl.
3. To the third sample just add 400 $\mu$ l of 2M HCl to form a sample blank.
4. Leave at room temperature for 1 hour for complete reaction of DNPH with free carbonyl groups.
5. Add 500 $\mu$ l trichloroacetic acid (4% w/v) to precipitate the protein.
6. Centrifuge at 14000g for 5 minutes.
7. Wash the pellet 4 times in 1:1 ethanol/ethyl acetate (v/v); at each wash vortex to break up the pellet, allow to stand for 10 minutes and then reform the pellet by centrifugation (14000g for 3 minutes).
8. Keep the supernatant of each wash to check the removal of free DNPH by its absorbance at 379nm.
9. After washing the pellet, add 0.6ml 6M guanidine hydrochloride in 20mM phosphate buffer, adjusted to pH 2.3 with trifluoroacetic acid and vortex.

10. Freeze at -20°C overnight and thaw, this allows the pellet to fully break up and dissolve in the guanidine hydrochloride.
11. Pipette duplicate 200µl aliquots of the solution of protein in guanidine hydrochloride to a microplate, and measure the absorbance at 379nm. The microplate colorimeter used was a Bio-tek microplate autoreader EL311, (Luminar technology, Hampshire).

The moles of protein-bound DNPH and thus the moles of carbonyl groups can be calculated directly from the absorbance using the molar extinction coefficient of 2,4-dinitrophenylhydrazine (22000 M<sup>-1</sup> cm<sup>-1</sup> [170]). The calculation is as follows;

$$[\text{DNPH or Carbonyl}] = \frac{(\text{nett})A_{379}}{\epsilon \times l} \text{ according to Beer - Lambert law}$$

$$\epsilon = 22000 \text{ M}^{-1} \text{ cm}^{-1}$$

$$l = 0.7 \text{ cm}$$

$$(\text{nett})A_{379} = (\text{sample}) A_{379} - (\text{sample blank}) A_{379}$$

sample blank = sample that has undergone all steps of the assay except reaction with DNPH.

$$\text{Carbonyl concentration in mmol ml}^{-1} = \frac{(\text{nett})A_{379} \times 10^3}{22000 \times 0.7}$$

but there is a 1 in 6 dilution due to the addition of guanidine hydrochloride thus

$$\text{Carbonyl conc. in mmol ml}^{-1} = \frac{(\text{nett})A_{379} \times 6}{15.4}$$

$$\text{Carbonyl conc. in mmol ml}^{-1} = (\text{nett})A_{379} \times 0.39$$

#### 4.3.2. *In vitro* glycation of nBSA and dBSA

Delipidated BSA (dBSA) purchased from Sigma (Sigma, Pool, Dorset, UK.) was produced by reaction of native BSA (nBSA) with charcoal by the method of Chen (1967); this is said not to cause denaturation [171]. Comparison of dBSA was made with nBSA of the same level of purification (fraction V). nBSA and dBSA were glycated for up to 21 days in phosphate buffer, assayed according to the methods described in chapter 2. and in addition assayed with DNPH as described above.

#### 4.3.3. Sugar-free re-incubation

BSA fructated to a range of extents (0.05M for up to 21 days) was separated from free fructose by exhaustive dialysis versus phosphate buffer (see page 57). Upon completion of dialysis the samples of fructated BSA were re-incubated at 37°C in sugar-free phosphate buffer for up to 15 days, assayed according to the methods described in chapter 2. and in addition assayed with DNPH as described above.



## 4.4. Results

### 4.4.1. DNPH assay development

**Calculation of the path-length ( $l$ )** of the microplate was necessary because the absorbance is measured through the meniscus and thus the length of the light path is dependent on the viscosity and volume of the liquid in the well. The curvature of the meniscus was taken into account by reading the average absorbance of three measurements taken at set distances from the centre of the well. The path-length was calculated for set volumes of liquid in the microplate as follows. The concentration of a BSA solution (in phosphate buffer) was calculated from its  $A_{280}$  (see page 58). This BSA was then stained using the bicinchoninic protein kit assay and the molar extinction coefficient ( $\epsilon$ ) of the complex was calculated (using  $A=\epsilon cl$ ) from its absorbance at 570nm. Varying volumes, of this stained BSA, were loaded onto a microplate and their absorbance at 570nm measured. The path length was calculated (again using  $A=\epsilon cl$ ) using the known concentration of BSA and its molar extinction coefficient when stained using the bicinchoninic protein kit assay. Figure 4.4.1.1. shows the linear relationship between path-length and the volume of liquid in the well; 200 $\mu$ l in this case gave an average path-length of 0.7cm across the meniscus.

**Absorbance spectra** of fructated protein after undergoing the DNPH assay are shown in figure 4.4.1.2. The left hand spectrum shows a fructated sample that has undergone the DNPH assay, the lower line is the sample blank and the upper line is after reaction with DNPH; both samples are in guanidine hydrochloride solution. The right hand difference spectrum (calculated from the left spectrum) represents the absorbance of protein-bound DNPH, illustrating a  $\lambda_{\max}$  at 375nm. Note that the slight blip in the spectrum of DNPH at 376 to 378nm is due to



experimental error, this is the region in which the Cecil 4000 spectrophotometer switches lamps to measure absorbance either in the UV or visible ranges.

**Removal of excess DNPH** from the protein is essential to increase the sensitivity of the assay. Figure 4.4.1.3. shows the effectiveness of the ethanol-ethyl acetate wash by its absorbance at 379nm. After 3 washes little more DNPH was removed and hence, in the case of BSA, 4 washes were adequate to remove unreacted DNPH.

**Loss of protein** during the assay was a concern; Levine *et al* (1990) reported that albumin was partially soluble in ethanol/ethyl acetate [172]. The concentration of total BSA (glycated and non-glycated) was measured before and after DNPH assay and showed a recovery of 99.7%. For the study of BSA in this thesis the loss of protein was therefore assumed to be negligible, but it should be checked for each protein studied. The results are summarised in the table below;

	Before DNPH assay	After DNPH assay
Mean protein concentration (mg ml <sup>-1</sup> )	39.9	39.8
number of samples	8	96
standard error from mean (± mg ml <sup>-1</sup> )	1.8	1.3

**The lower limit of linearity** of the absorbance at 379nm is shown in figure 4.4.1.4. BSA was fructated (0.05M fructose) for 8 days and diluted to various concentrations into phosphate buffer containing non-glycated BSA, such that the overall protein concentration in each sample was 40mg ml<sup>-1</sup>; hence the protein



concentration remained the same, but the proportions of fructated BSA varied. The DNPH assay was then used to test for carbonyl groups in the range of samples.

The inter-assay coefficient of variation is shown for a range of samples in figure 4.4.1.5. The figure describes the formation of protein-bound carbonyl groups on BSA incubated either with 50mM fructose or on its own over 8 days. There is a clear increase in protein-bound carbonyl groups due to fructation which forms a plateau after 5 days. The error bars on the figure are the standard error of the mean of 8 separate assays, while the figures represent the inter-assay coefficient of variation. A benchmark of acceptable coefficient of variation is <5%, which is easily met by all the samples that have been fructated; but the results for day 0 and all the results for the sugar-free control can only be used as an indicator of trend, not as an accurate indicator of absolute quantity.

Freezing and thawing the protein pellet in 6M guanidine hydrochloride breaks it up into smaller particles allowing a greater surface area to volume ratio for the solvent to act on. Without the freezing step, a great deal of protein remains as precipitate even after 15 minutes boiling in 6M guanidine hydrochloride. Figure 4.4.1.6. shows the results of the same samples of BSA incubated with 200mM glucose for up to 18 days with and without the freezing step, the figure clearly shows a greater yield with the step than without.



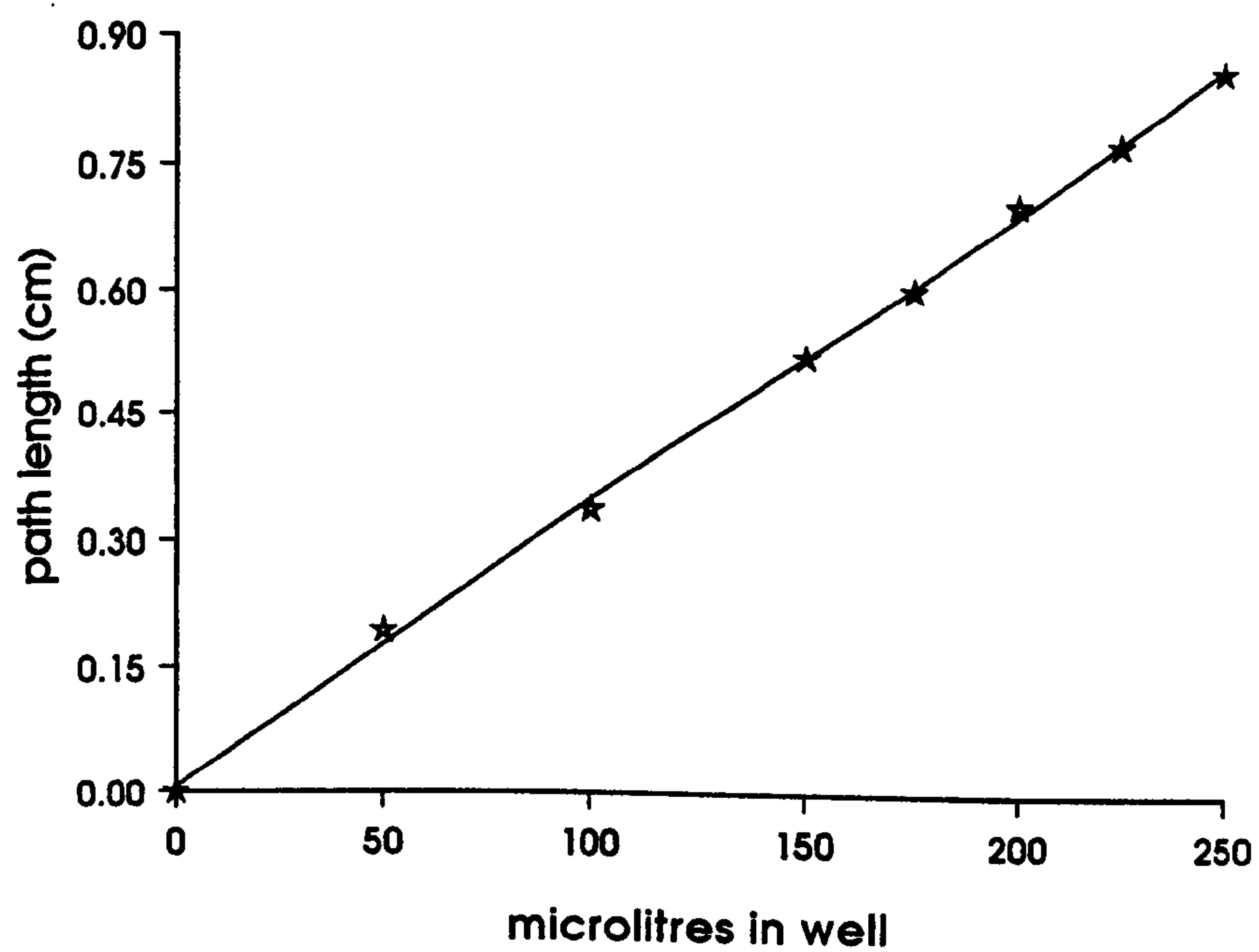


Fig. 4.4.1.1. The path-length is proportional to volume of liquid in a microplate well.

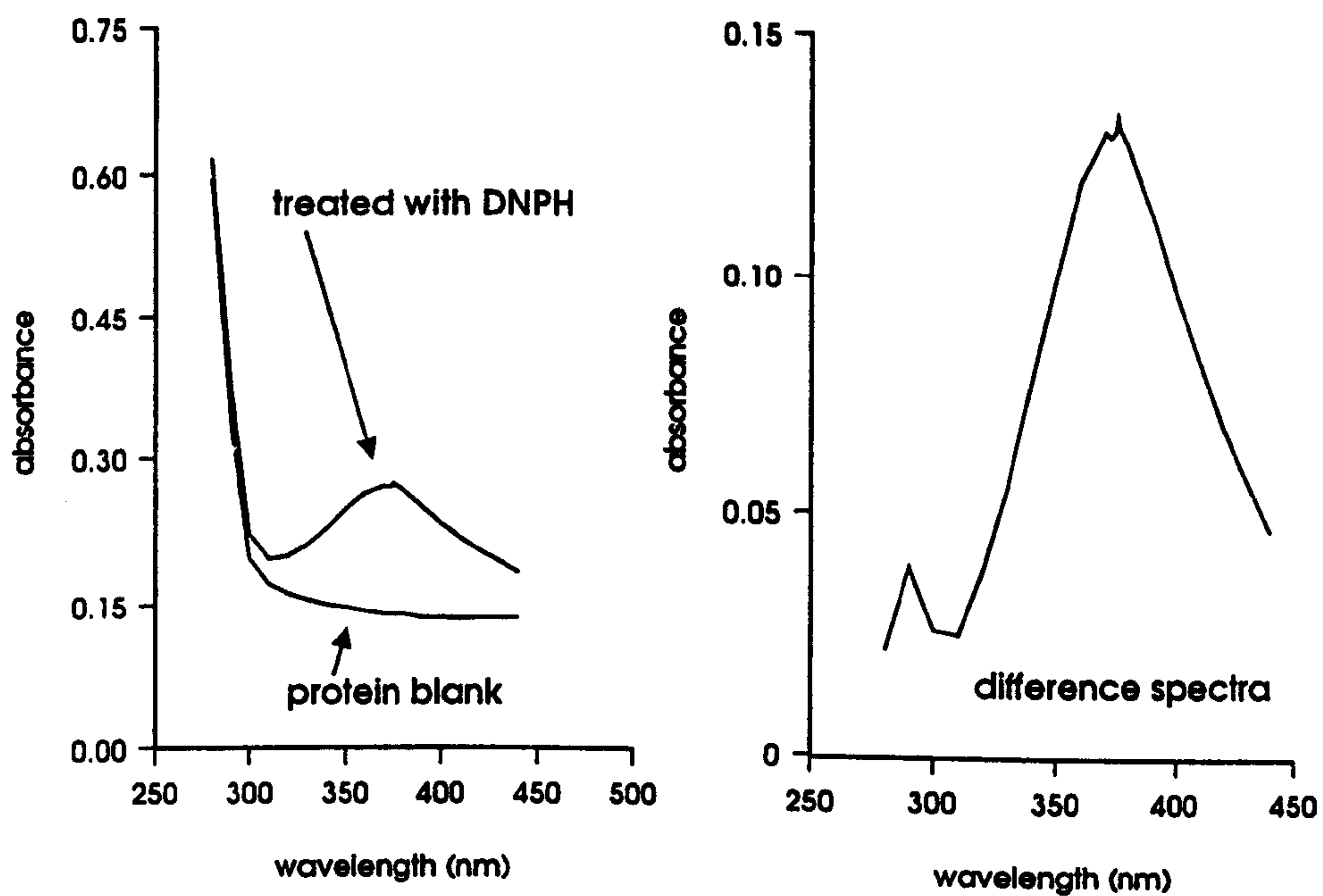


Fig. 4.4.1.2. Left; absorbance spectra of BSA fructated for 21 days with 0.005M fructose, upper line is reacted with DNPH, lower line is its sample blank. The right hand graph is the difference spectra of the spectrum on the left; i.e. net absorbance due to the 2,4-dinitrophenylhydrazone-protein adduct.

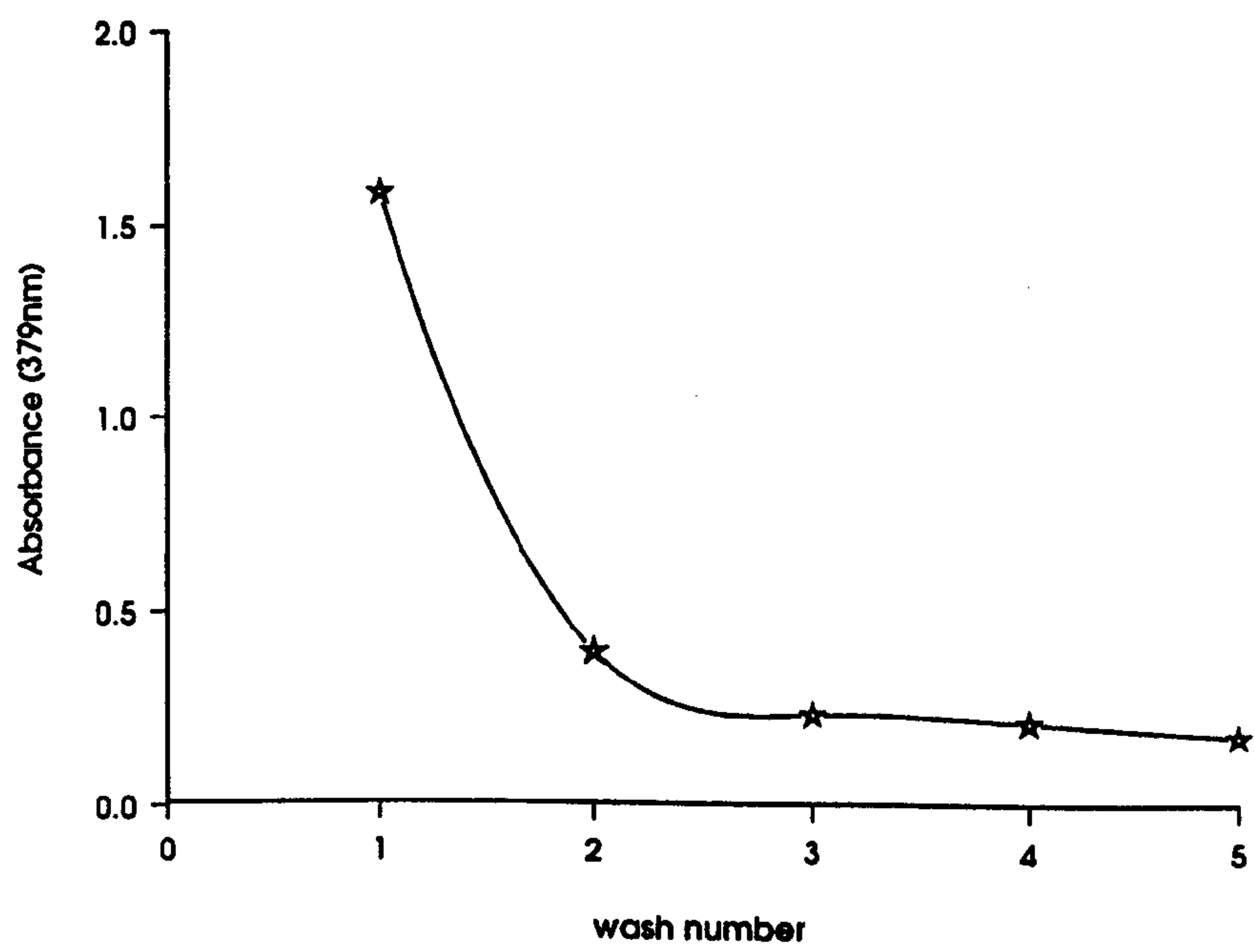


Fig. 4.4.1.3. Typical graph of the DNPH content of ethanol/ethyl acetate (1:1) washes.



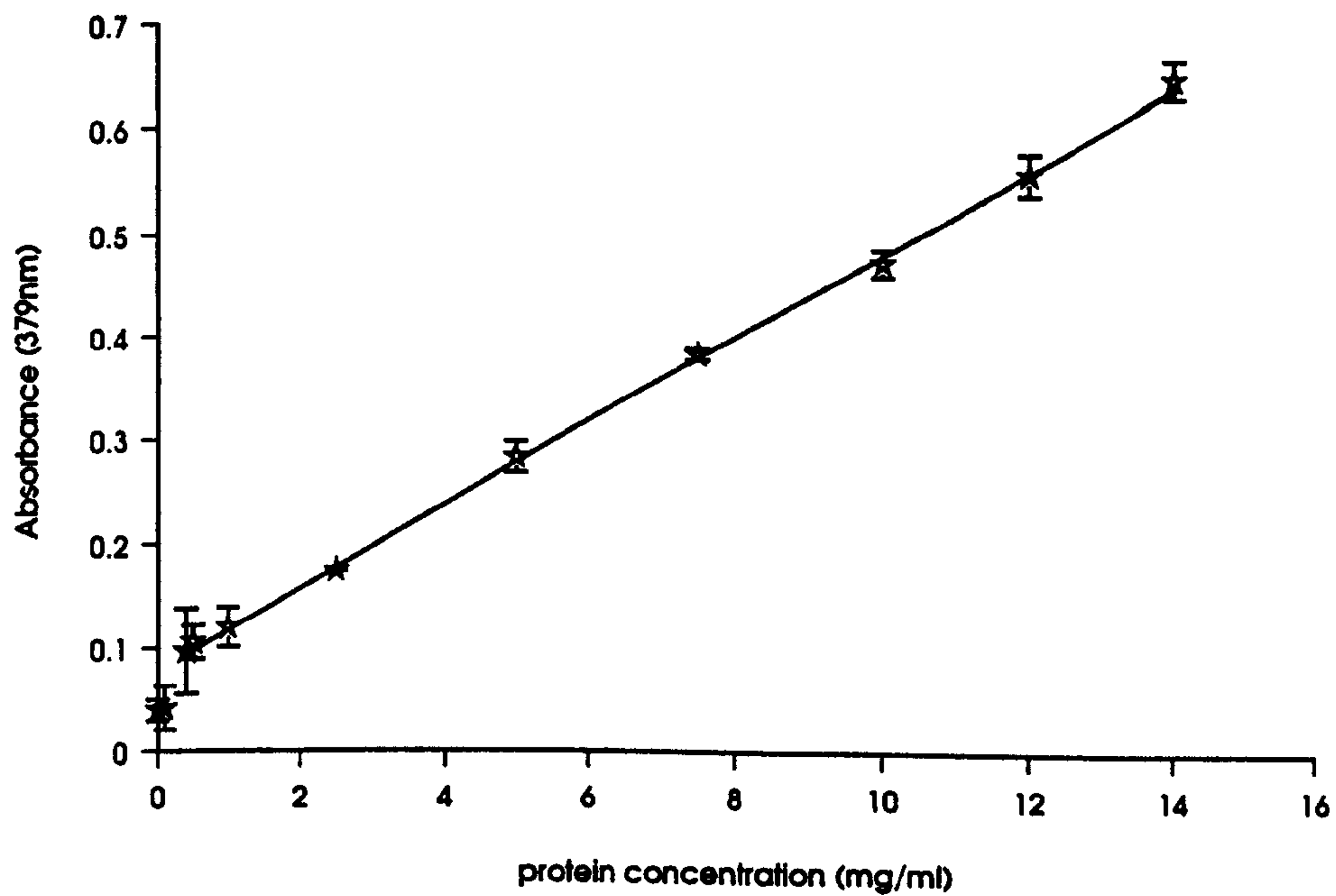


Fig. 4.4.1.4. The concentration range in which protein-bound DNPH is directly proportional to its absorbance at 379nm. Fructated BSA was prepared by 8 days incubation with 0.05M fructose followed by dilution into non-glycated BSA.

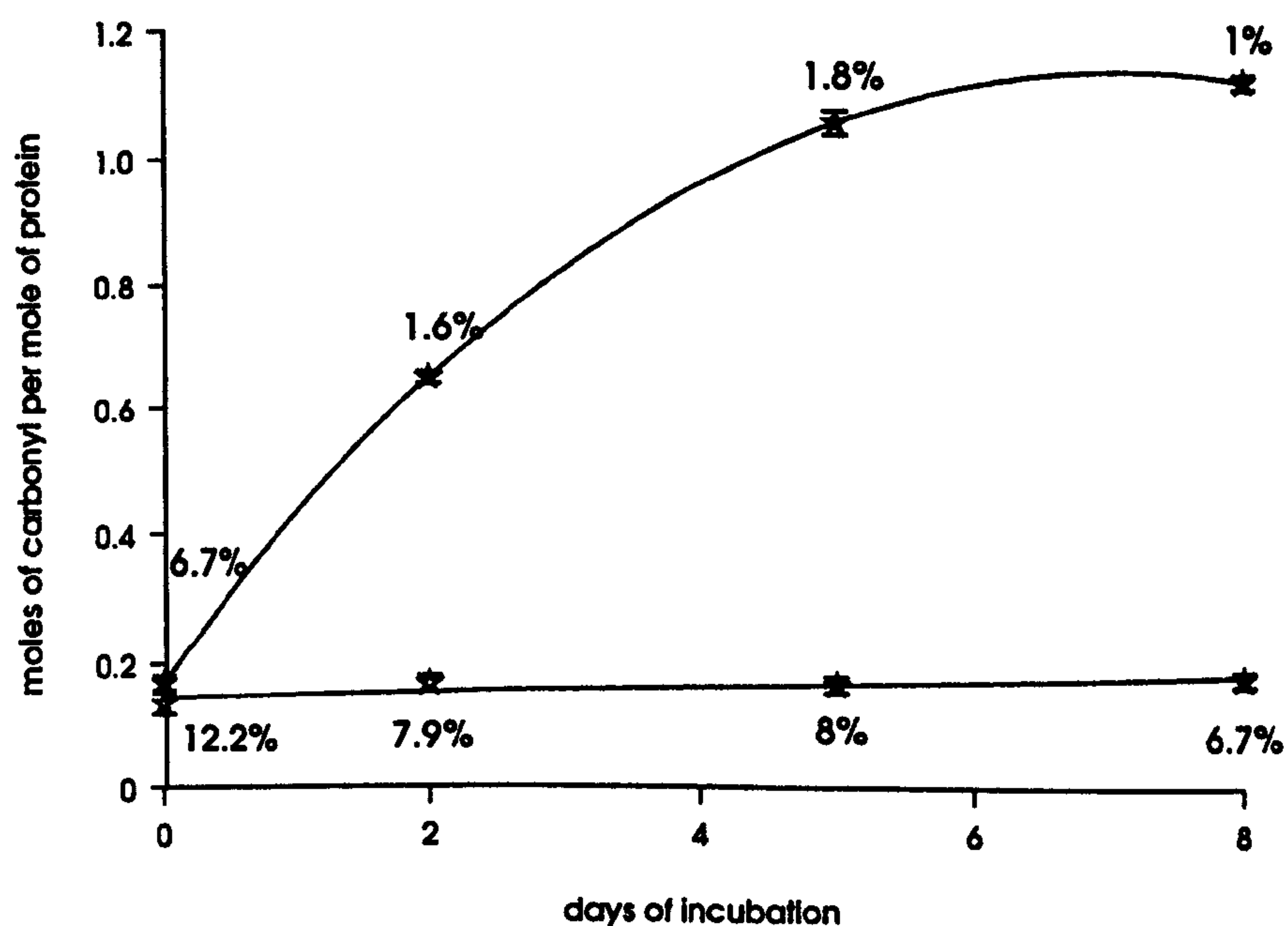


Fig. 4.4.1.5. Comparison of the protein-bound carbonyl content of fructated BSA (upper line, 0.05M fructose included) with its sugar-free control (lower line). The DNPH assay was carried out 8 times; the error bars represent the standard error from the mean of those assays, while the figures are the inter-assay coefficient of variation of the samples.

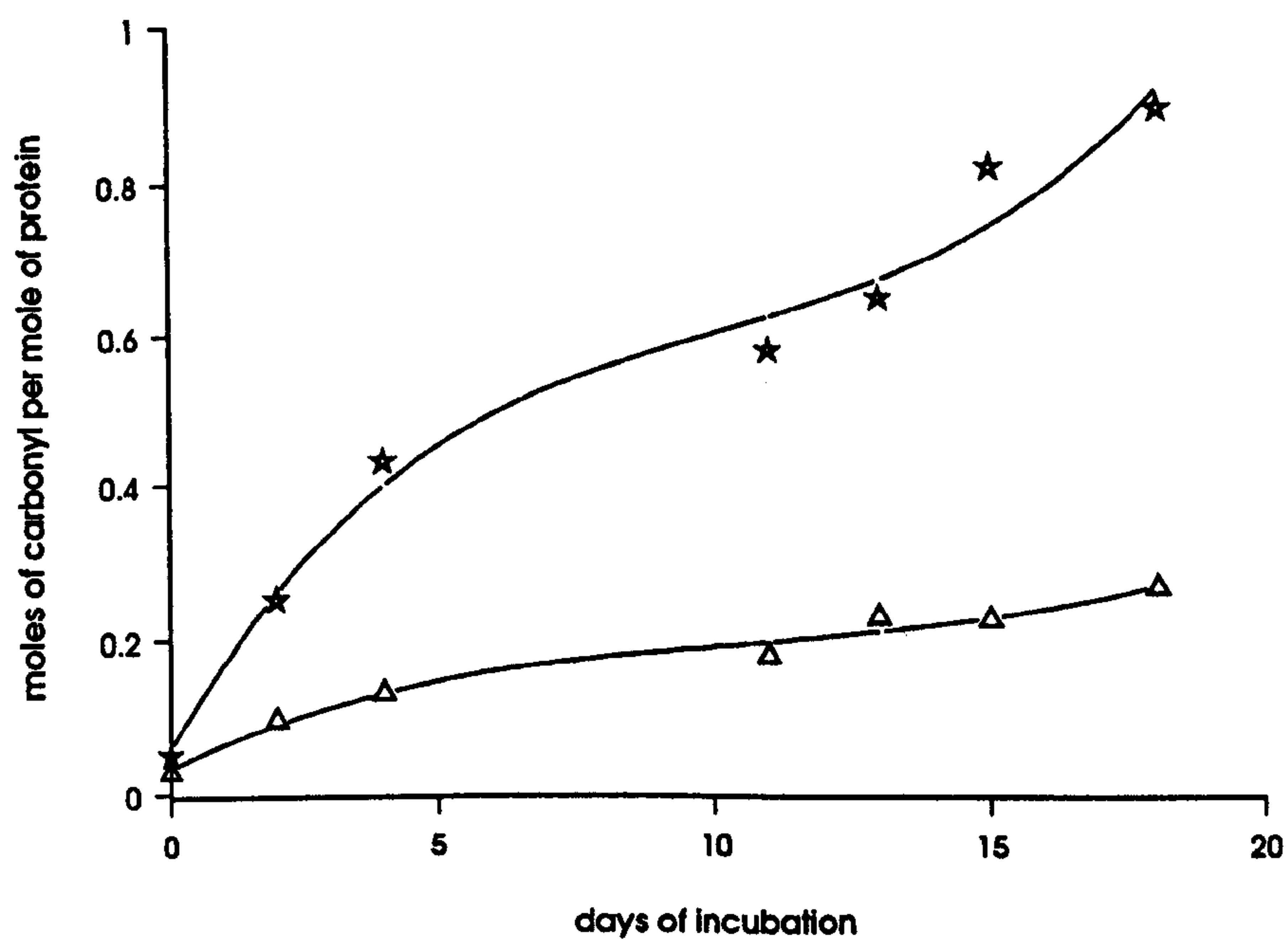


Fig. 4.4.1.6. The freezing step increases the yield of protein-bound DNPH by breaking up the protein pellet, allowing greater solvation. The upper line is with the final freezing step of the assay while the lower line is without. Glycated BSA was prepared by up to 18 days incubation with 0.2M glucose.



#### 4.4.2. The effect of lipid

The previous section showed that the production of protein-bound carbonyl groups was dependent on glycation (Fig. 4.4.1.5.); i.e. there was very little change in the sugar-free control. This section investigates the effect of lipid on protein-bound carbonyl production and relates it to AGE-fluorescence. Peroxidation of lipids and AGE formation occurs *in vivo* under similar conditions; both mechanisms are essentially oxidative in nature and lead to development of similar fluorescence spectra [160 and 162]. Products of lipid oxidation have been reported to react with Amadori product, enhancing the production of AGE-chromophores; also, lipid oxidation has been reported to be enhanced in the presence of AGEs [163 and 164]. Thus the production of lipid peroxides and AGEs is interactive, with the production of one influencing the production of the other. The DNPH assay detects carbonyl compounds that are TCA and ethanol/ethyl acetate insoluble. By comparing glycated native and delipidated protein the influence of lipid on the Maillard reaction can be investigated.

Figures 4.4.2.1. through to 4.4.2.3. compare glucation and fructation using various measures of glycation and AGE formation, and illustrate the effect of lipid on these measures. Please note that graphs of the sugar-free controls have been omitted from these figures for clarity. There was no increase in any of the measures of glycation, unless monosaccharide was also present during incubation (e.g. Figs. 4.4.3.1., 4.4.1.5. and 4.4.3.3.). The assay results of the sugar-free controls have been subtracted from the results of the glycated protein, to enable comparison of glycation on the different proteins.

The periodate assay measures formaldehyde released from hexose upon periodate oxidation. Amadori product is assumed to be the major product of glucation that

produces a positive result. The minor ketonic Heyns product will also produce periodate positive material (PPM) according to Malprades' rules [14]. The higher initial level of PPM in the dBSA compared with the nBSA is probably due to differences in the starting material from which the proteins were purified from. Figure 4.4.2.1. illustrates the formation of Amadori product upon glucation of nBSA and dBSA. The figure shows that while dBSA exhibits more Amadori product per mole of protein than nBSA, the rate of glucation is similar in both proteins. Fructation of BSA produces much less PPM than glucation, and fructation of delipidated BSA produces less PPM than the native form.

In contrast to the production of PPM, fructation of nBSA produces more protein-bound carbonyl groups and pentosidine-linked fluorescence than glucation (Figs. 4.4.2.2. and 4.4.2.3.). This occurred even though the concentration of fructose used for preparation of the samples used in the DNPH assay was ten fold lower than that of the periodate assay.

Figure 4.4.2.2. also indicates that in contrast to the production of PPM, the production of protein-bound carbonyl groups is not as great on the delipidated BSA than on the nBSA. In fact there is very little production of protein-bound carbonyl groups on dBSA when glycated by either monosaccharide. The slightly lower rate of production of PPM upon fructation (Fig. 4.4.2.1.) suggests that, if this is a true effect, then PPM production, from fructation of BSA, is at least in part dependent upon the presence of lipid. The production of PPM from fructose is an area of the work presented in this thesis that deserves more attention, if more time had been available I would have liked to have investigated the effect seen in figure 4.4.2.1. more thoroughly.

The development of pentosidine-linked fluorescence (Fig. 4.4.2.3.) upon glycation is very similar to the production of protein-bound carbonyl groups on the same



samples (Fig. 4.4.2.2.). Fructation of nBSA produced more fluorescence than glucation even though the concentration of glucose was 40 times that of fructose. Delipidated BSA developed very little fluorescence upon glycation when compared to its native counterpart, this indicates that the lipid component of BSA is involved in the production of AGEs.

A preliminary experiment in which nBSA was delipidated after fructation, showed that the DNPH assay detects carbonyl groups produced both on the protein and the lipid components of BSA (results not shown). The experiment used the method of Chen (1967) to delipidate the protein, so that the results would be comparable to commercially produced dBSA [171]. The results indicated that, after 10 days glycation of BSA with 0.5M fructose, 60% of the carbonyl groups detected by the DNPH assay were present on the lipid fraction of the protein. After 21 days the total carbonyl detected had increased, but the proportion on the lipid component had decreased to 30%, indicating that the majority were present on the protein component of the BSA.

The results of this section indicate that while fluorescent AGEs are dependent on the presence of lipid, Amadori product is unaffected. The production of protein-bound carbonyl is also dependent on lipid, thus the DNPH assay must be detecting post-Amadori (and by analogy also post-Heyns) compounds.



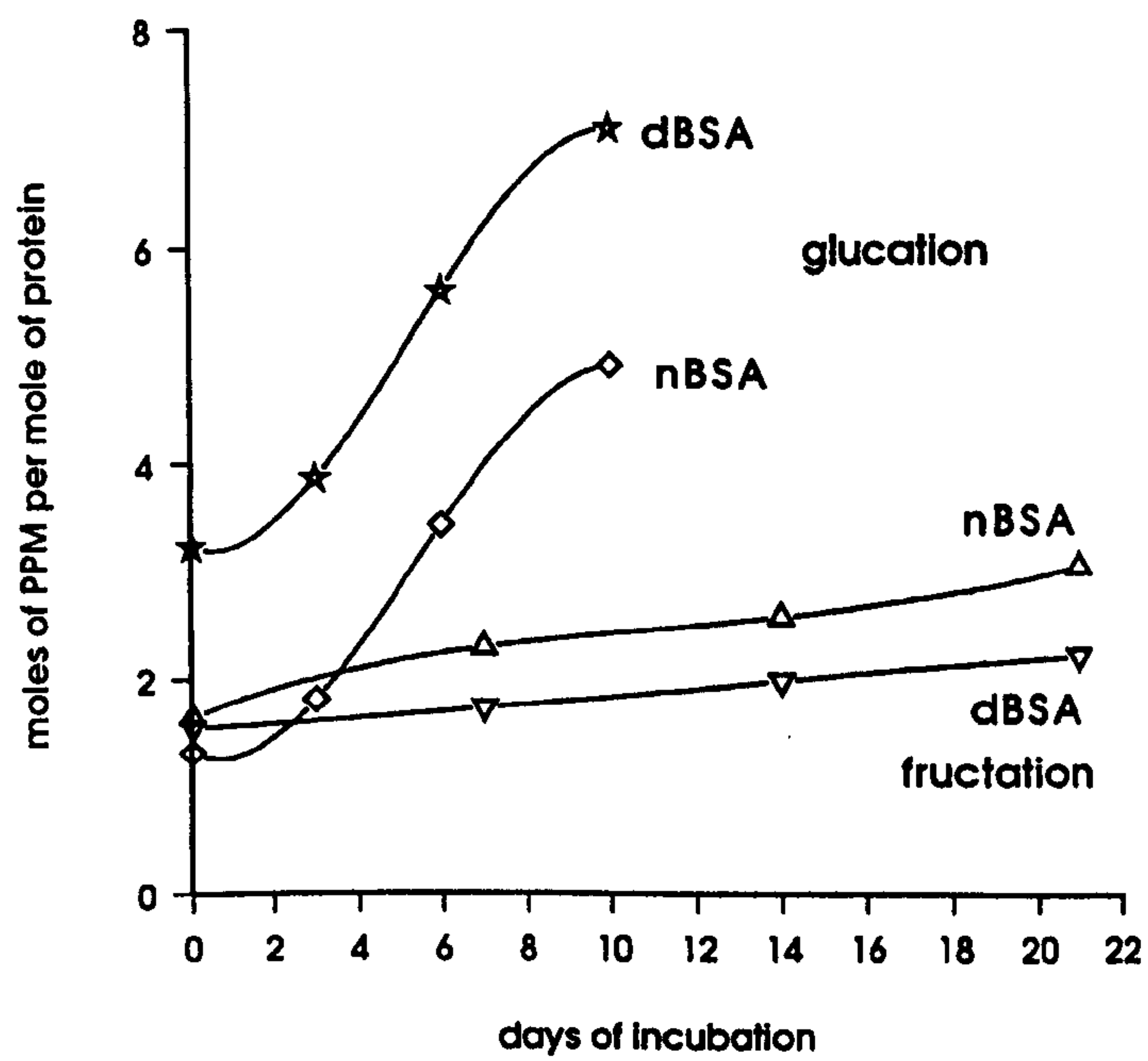


Fig. 4.4.2.1. Production of periodate positive material on nBSA and dBSA as a result of glycation by glucose (0.2M) or fructose (0.05M).

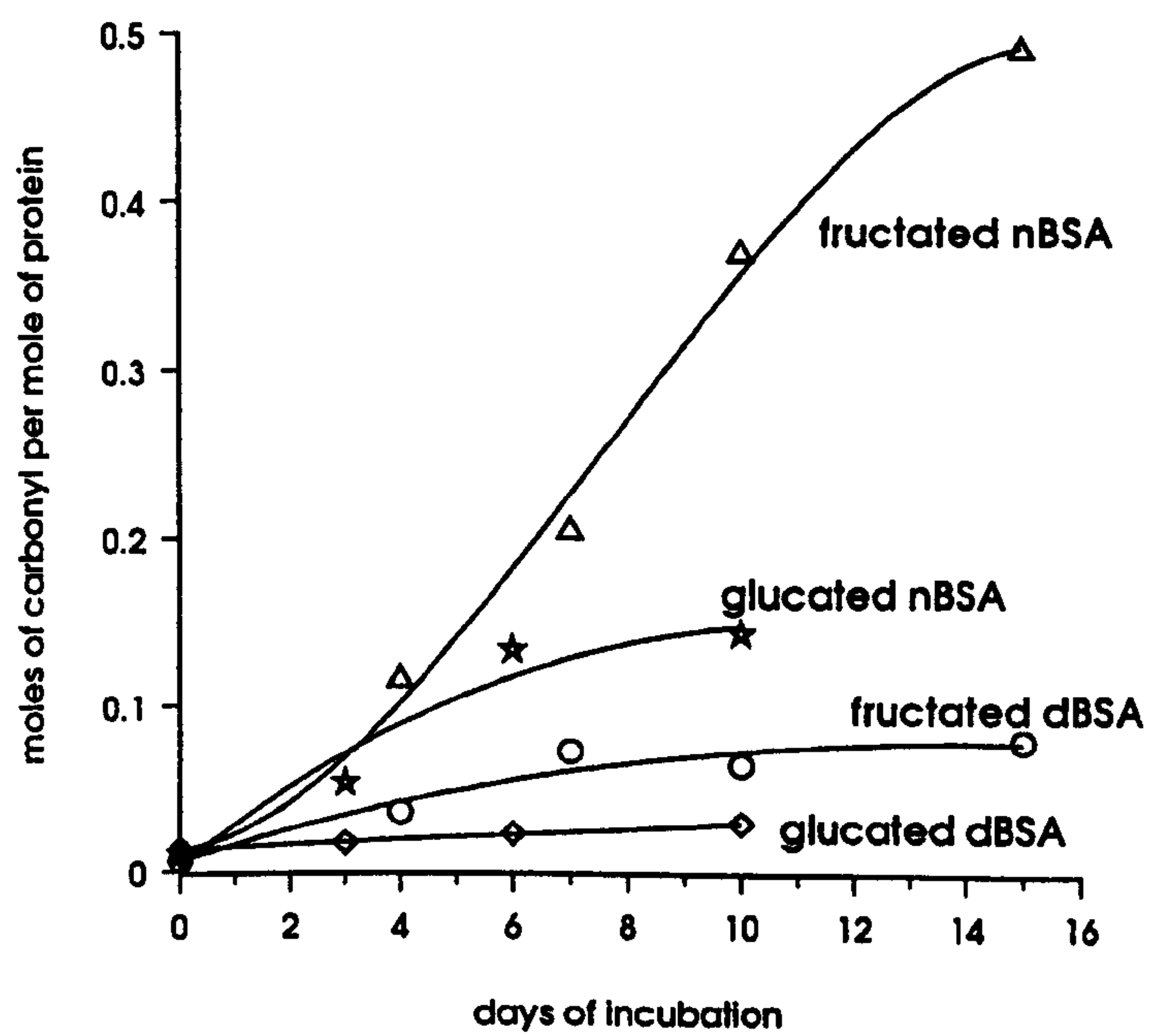


Fig. 4.4.2.2. Formation of protein-bound carbonyl groups on nBSA and dBSA upon glycation with glucose (0.2M) or fructose (0.005M).

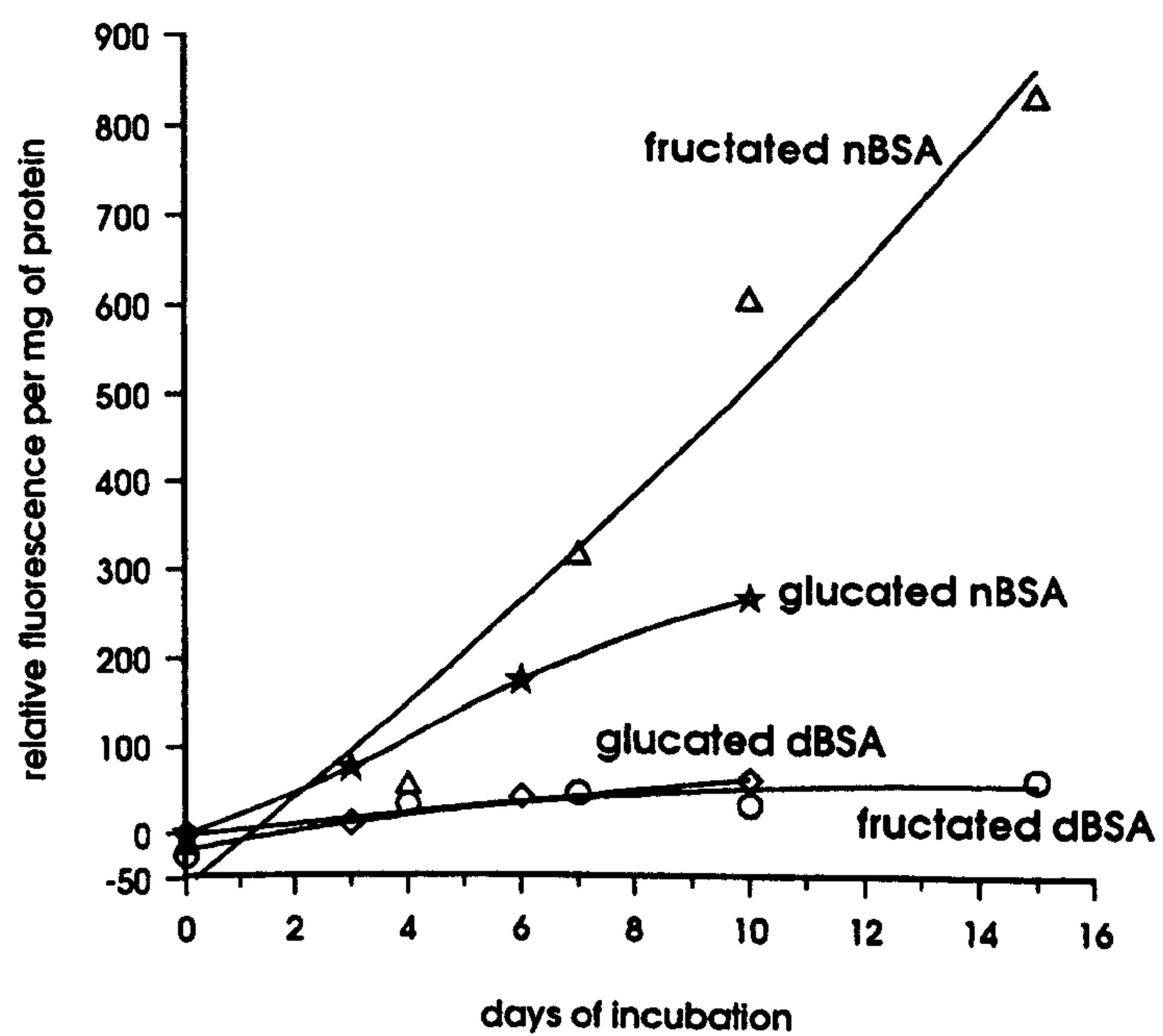


Fig. 4.4.2.3. Development of pentosidine-linked fluorescence of nBSA and dBSA upon glycation with glucose (0.2M) or fructose (0.005M).



#### 4.4.3. Inhibitors of AGE formation

Glycoxidation is a term that has been coined to highlight the link between metal catalysed oxidation (MCO) and AGE formation [134]. Amadori product formation is unaffected by factors that affect oxidation of proteins whereas AGE formation is dependent on MCO [179, 134 and 177]. This section of the chapter investigates the effect of DTPA and aminoguanidine on protein-bound carbonyl formation. DTPA is a metal chelator and its inclusion during glycation inhibits metal catalysed oxidation and thus AGE formation. Aminoguanidine is a nucleophilic hydrazine, that inhibits the production of AGEs, probably by reacting with the same compounds detected by DNPH [165].

The effect of aminoguanidine and DTPA on the various measures of glycation is illustrated in figures 4.4.3.1. through to 4.4.3.5. The first figure (4.4.3.1.) shows that the formation of Amadori product is promoted by the presence of aminoguanidine or DTPA. In contrast to this, formation of protein-bound carbonyl is partially inhibited and fluorescence completely inhibited by 50mM aminoguanidine (Figs. 4.4.3.2. and 4.4.3.3.). Similarly 1mM DTPA inhibits formation of protein-bound carbonyl groups and fluorescence (Figs. 4.4.3.4. and 4.4.3.5.).

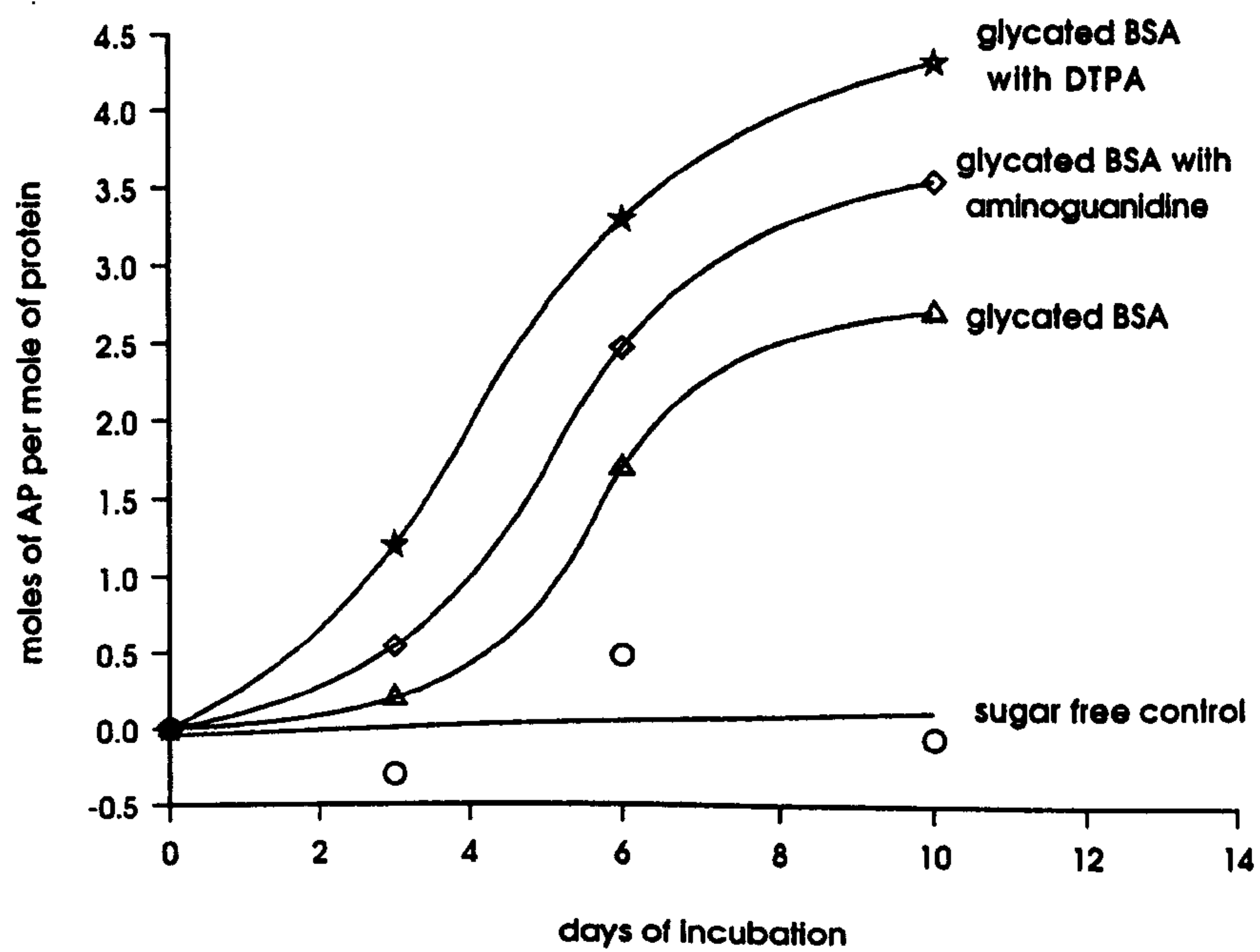


Fig. 4.4.3.1. Promotion of Amadori product formation in the presence of 50mM aminoguanidine or 1mM DTPA, upon glycation of BSA with 0.2M glucose.

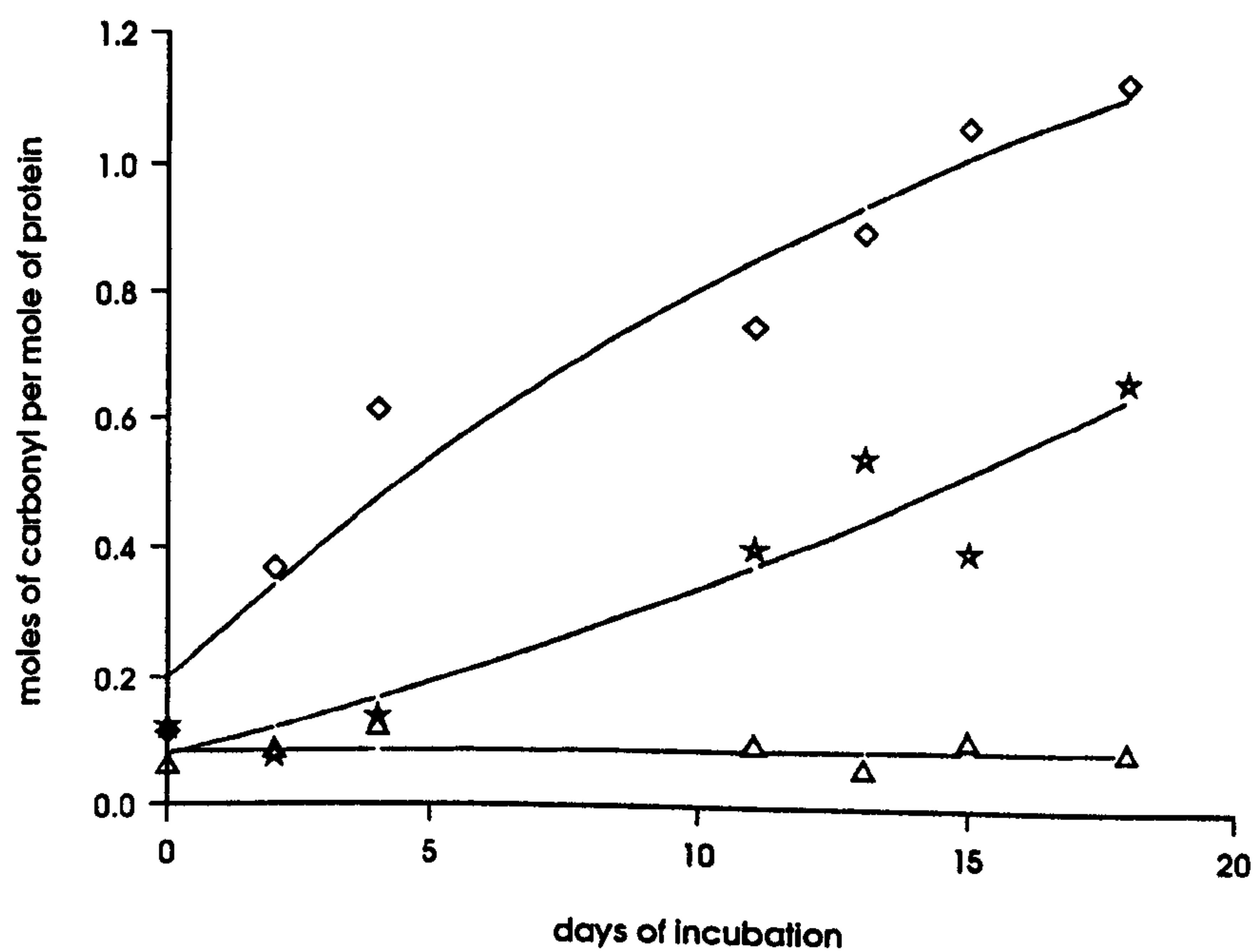


Fig. 4.4.3.2. Protein-bound carbonyl group formation of BSA incubated alone (triangle) or glucated (0.2M glucose) in the presence (star) or absence (diamond) of 50mM aminoguanidine.



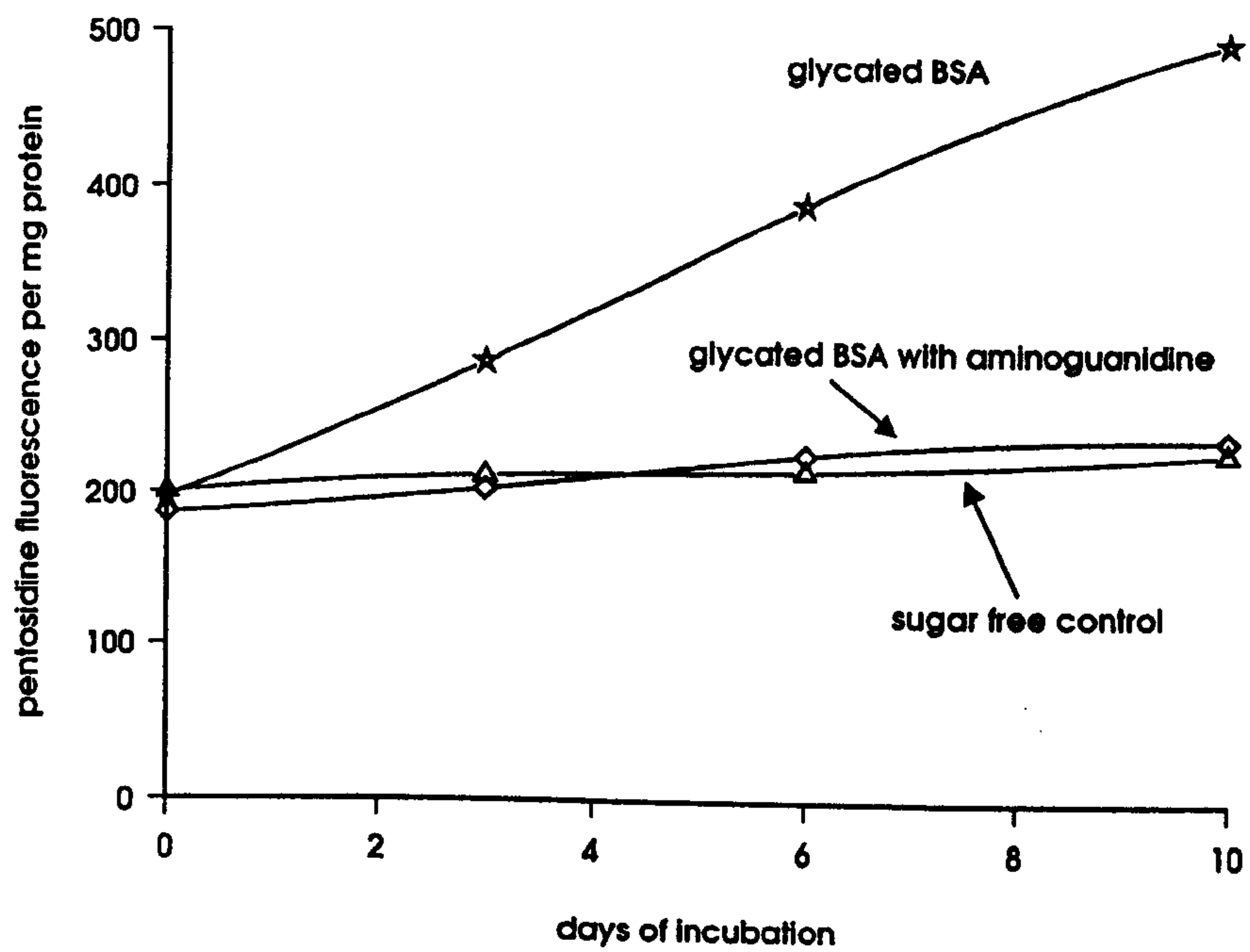


Fig. 4.4.3.3. Development of fluorescence is inhibited by 50mM aminoguanidine. Glycation of BSA was with 0.2M glucose.

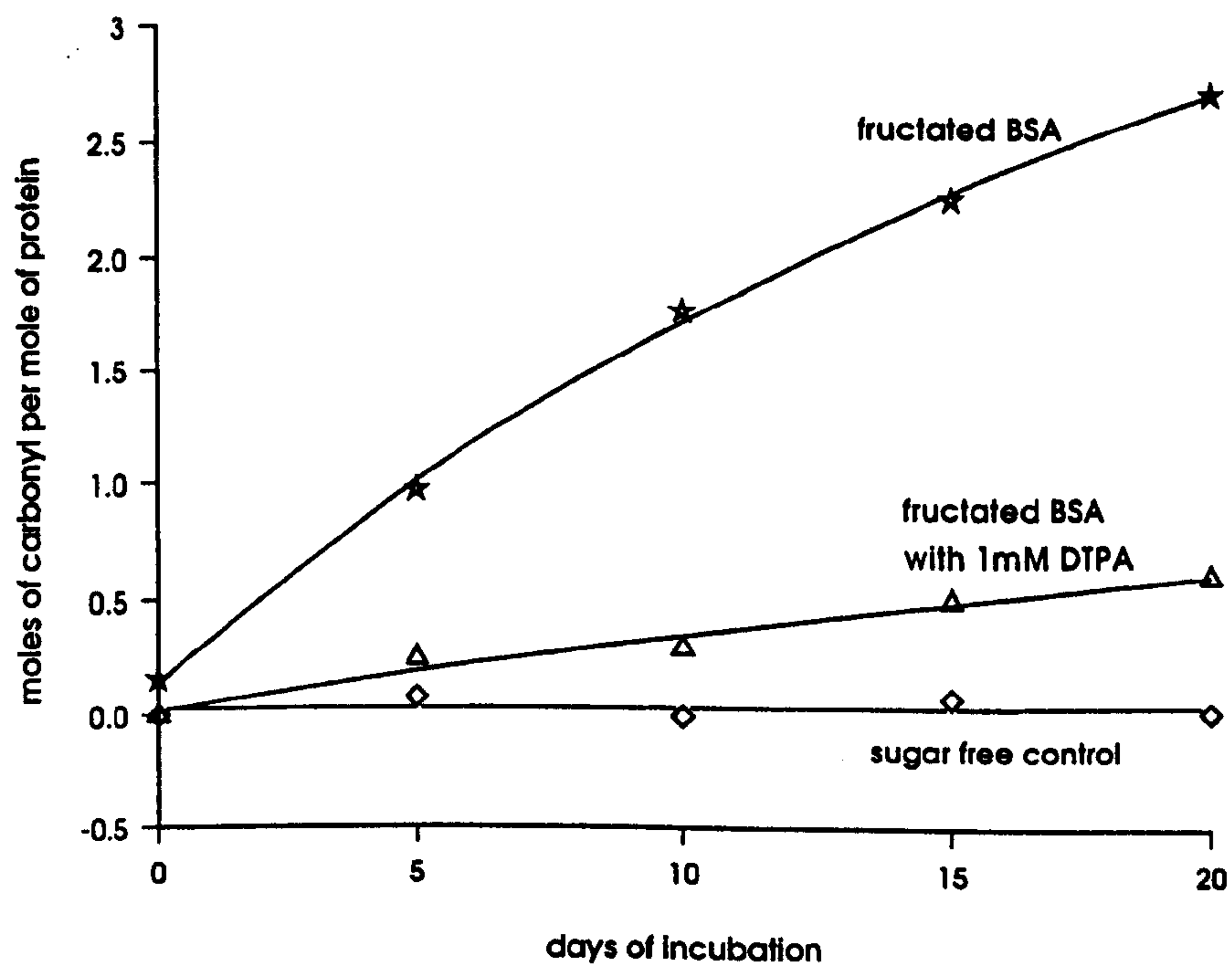


Fig. 4.4.3.4. Inhibition of protein-bound carbonyl formation on fructated BSA (0.5M fructose) by the metal chelator DTPA. Note that the moles of carbonyl per mole of protein is an underestimate because the absorbance was measured at 405nm and the freezing step was omitted.

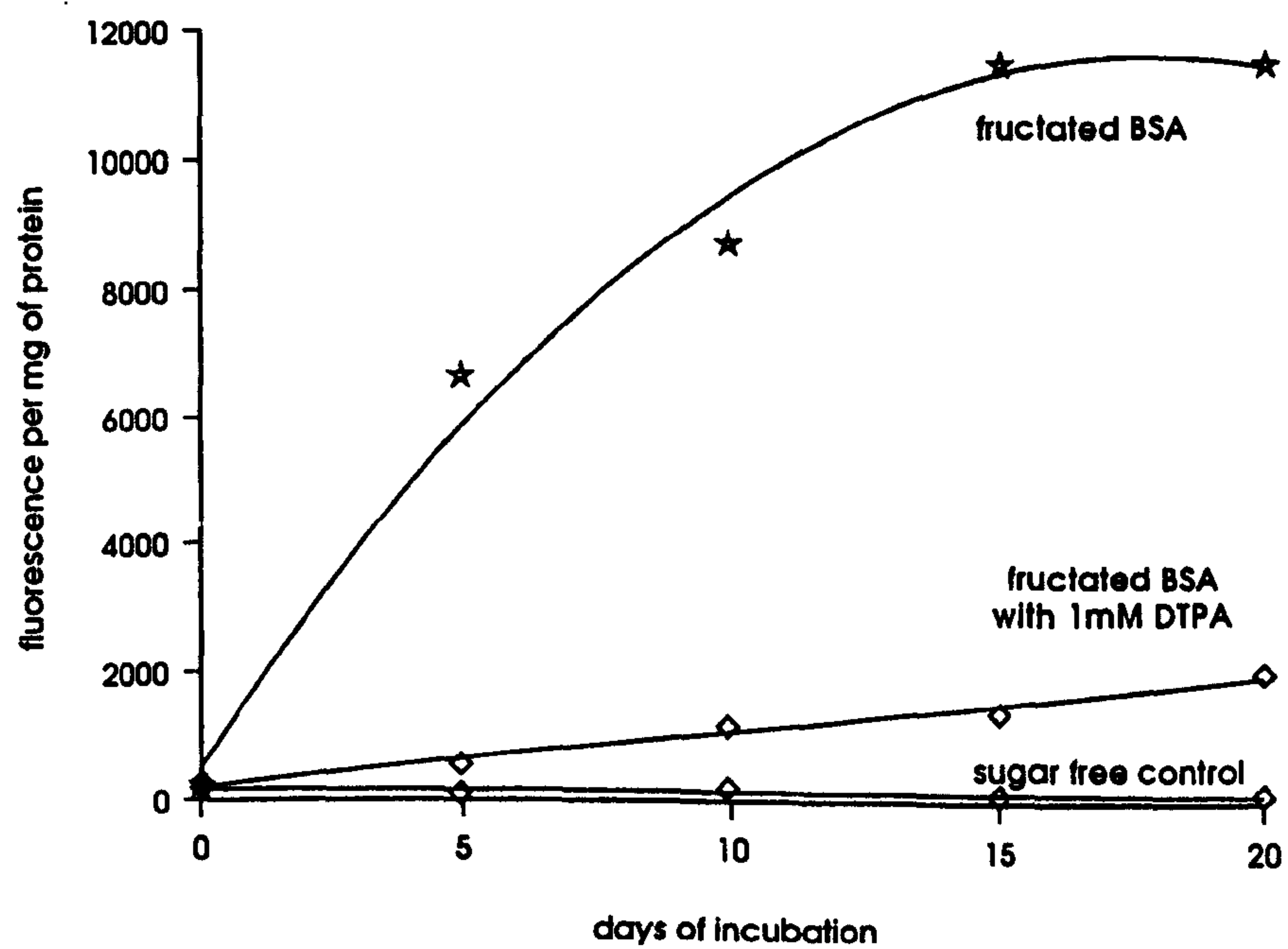


Fig. 4.4.3.5. Inhibition of fluorophore formation on fructated BSA (0.5M fructose) by 1mM DTPA.



#### 4.4.4. Order of the intermediates in the Maillard reaction

In the introduction of this thesis, figure 1.1. illustrated the Maillard reaction as we know it now, with two known intermediates to AGE formation (Schiffs base and Amadori or Heyns product). Part of this chapter is an investigation of the points at which the compounds that react with DNPH occur in the Maillard reaction. So far, I have shown that protein-bound carbonyl groups and fluorophore formation are dependent on free metal and lipid for their formation. Amadori product, on the other hand, is unaffected by free metal or lipid. These findings suggest that along with fluorescence the protein-bound carbonyl groups detected by DNPH are post-Amadori or post-Heyns products. This section of the thesis presents results of experiments designed to determine whether the protein-bound carbonyl groups detected are themselves end-products of the Maillard reaction or intermediates in the production of AGEs.

The figures of this section of the thesis illustrate the changes in the measures of glycation as a function of the time of sugar-free re-incubation of fructated BSA. The time of incubation with fructose is marked on the graphs, while the extent of fructation can be read off the y-axis. The points on the x-axis marked 'before re-incubation' represents the measurements assayed before dialysis; thus for figures 4.4.4.2. and 4.4.4.3. the change between 'before re-incubation' and 0 days of re-incubation, occurred during the 4 days of exhaustive dialysis.

The upper part of figure 4.4.4.1. shows the production of periodate positive material (PPM) during sugar-free re-incubation of fructated BSA. The lower part shows the same samples, but the PPM produced during the initial glycation has been subtracted, thus illustrates the changes during re-incubation only. The graphs in the lower part of figure 4.4.4.1. indicate that there is an increase in PPM that is

not due to fructation. Both non-glycated (day 0 sample) and fructated BSA show an increase in PPM that is proportional to the time of re-incubation, but is the same no matter for how long the BSA was initially fructated. This indicates that the increase of PPM seen with re-incubation is due to oxidation rather than post-Heyns reactions. The higher yield of PPM from fructated BSA than from the sugar-free control is probably a reflection of the oxidative damage to the protein caused by glycation.

The decrease in protein-bound carbonyl groups during re-incubation is illustrated in figure 4.4.4.2. With the exception of the samples that were initially fructated for 0 days, all the samples show a decrease in protein-bound carbonyl proportional to the period of re-incubation. The net decrease in protein-bound carbonyl groups during sugar-free re-incubation is illustrated by the lower part of figure 4.4.4.2. The figure shows that there is a decrease in protein-bound carbonyl groups that is proportional to the degree of initial fructation of the BSA (i.e. days of fructation).

Conversely fluorescence of fructated BSA increases, with the time of sugar-free re-incubation, in proportion to the time period of initial fructation (Fig. 4.4.4.3.). The lower part of figure 4.4.4.3. illustrates the net increase in fluorescence that occurred during sugar-free re-incubation, while the upper part of the figure also shows the fluorescence produced during fructation.

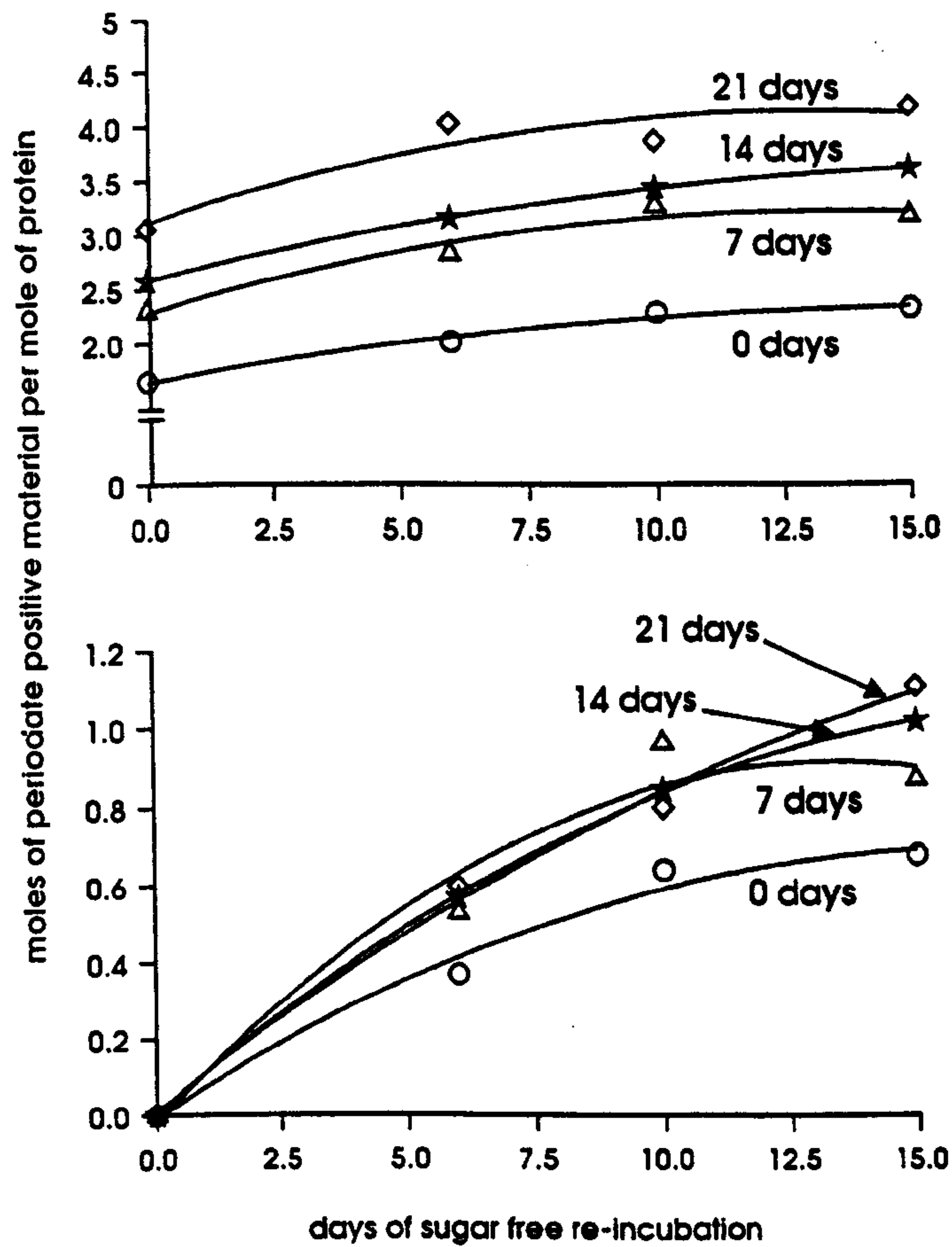


Fig. 4.4.4.1. Periodate positive material produced as a result of re-incubation of fructated BSA in sugar-free phosphate buffer. The days of initial glycation (0.05M fructose) of the BSA are indicated by the figures on each curve. Upper; the total increase in PPM during fructation and sugar-free re-incubation. Lower; net increase in PPM during sugar-free re-incubation only.



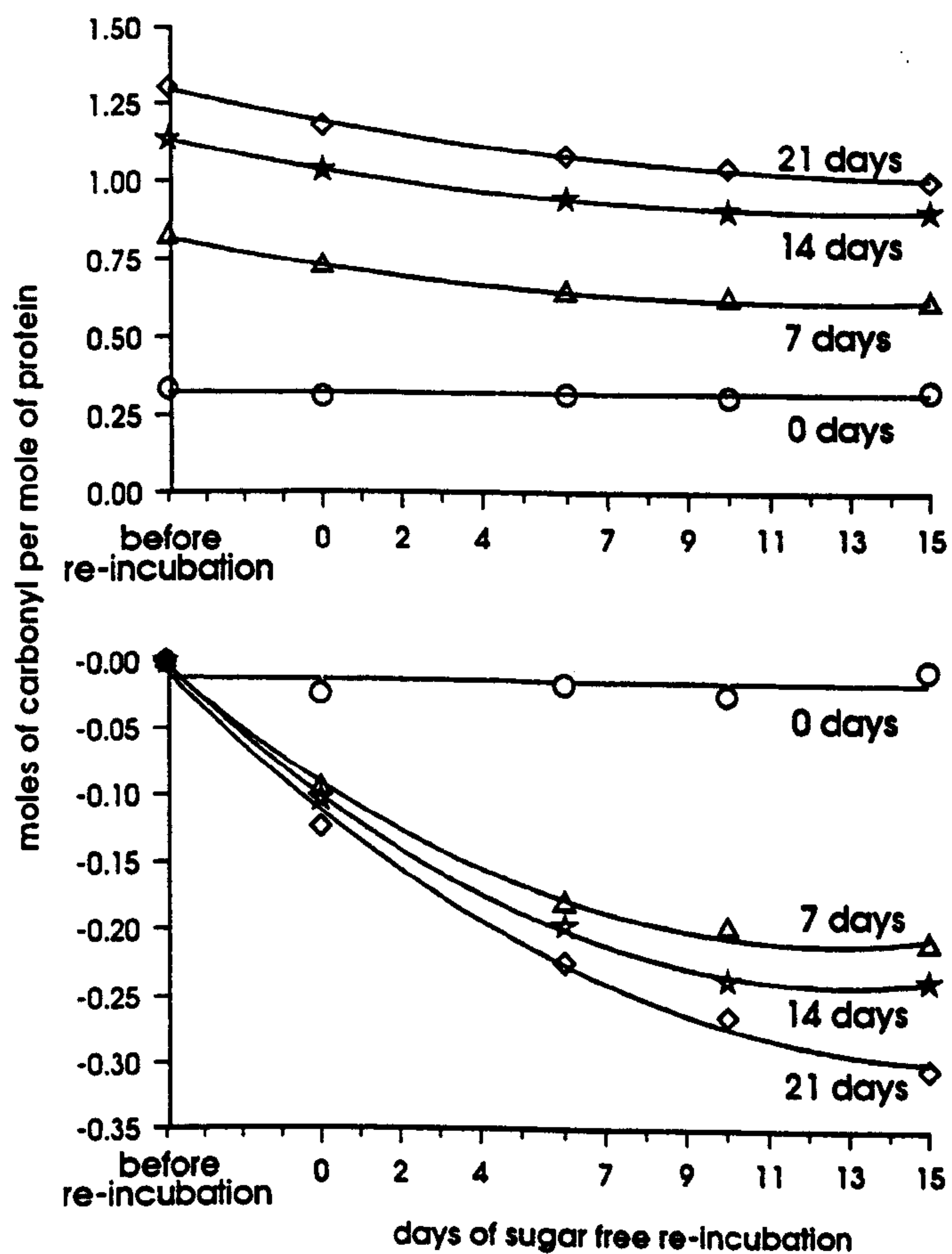


Fig. 4.4.4.2. Loss of protein-bound carbonyl due to sugar-free re-incubation. The days of initial glycation (0.05M fructose) of the BSA are indicated by the figures on each curve. Upper; the increase in protein-bound carbonyl groups during fructation is illustrated by the points marked 'before re-incubation,' followed by their subsequent decrease as a function of the time of sugar-free re-incubation. Lower: net decrease in protein-bound carbonyl groups during sugar-free re-incubation.

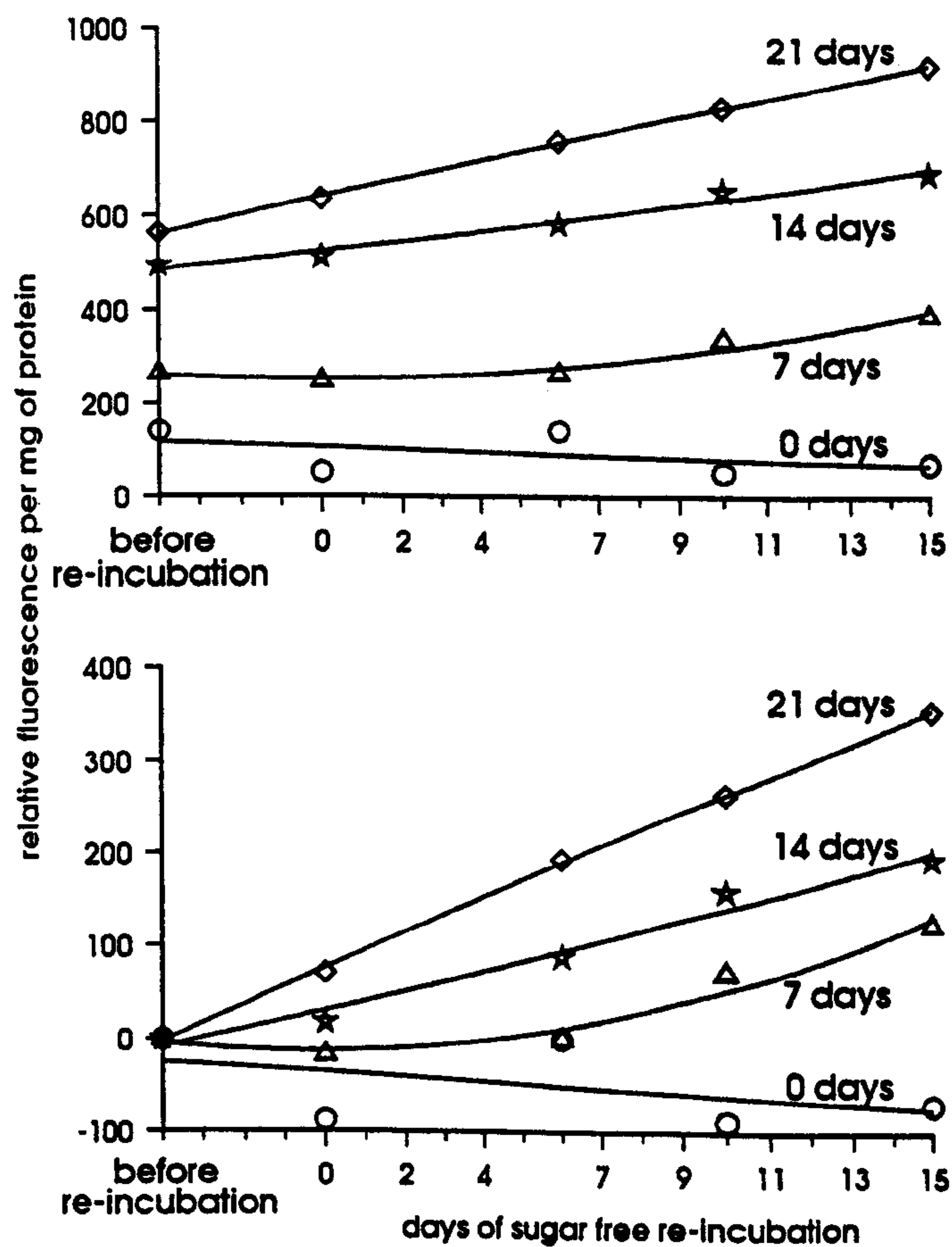


Fig. 4.4.4.3. Increase in pentosidine-linked fluorescence with time of re-incubation. The days of initial glycation (0.05M fructose) of the BSA are indicated by the figures on each curve. Upper; total increase in fluorescence during fructation (points marked 'before re-incubation') and sugar-free re-incubation. Lower: net increase in fluorescence during sugar-free re-incubation.

## 4.5. Discussion

Ahmed and Furth (1992) reported that the common assays for glucation failed to detect fructation and that the higher AGE-fluorescence of fructation indicated that its role *in vivo* was being underestimated [209]. Detection of fluorescence is not a quantitative technique; some fluorophores may fluoresce more than others, different proteins and sugars may form different proportions of different AGEs and some proteins will quench more than others. A principle aim of this thesis was to develop a quantifiable assay for protein fructation and glycation by other sugars.

### 4.5.1. The DNPH assay

The reaction of hydrazines, specifically 2,4-dinitrophenylhydrazine, with aldehydes and ketones is an important method used for their detection and has also been used with varying success for the detection of glycation; see the introduction of this chapter for a brief review. A method reported by Levine *et al* (1990), using DNPH for detection of oxidation of protein, has here been adapted to detect fructation [170]. To decrease the assay time, the absorbance was measured using a microplate reader (colorimeter), which measures absorbance through the meniscus and will therefore vary with the height of the liquid in the wells of the microplate. Calculation of the moles of protein-bound DNPH utilises a known molar absorption coefficient and the Beer-Lambert law ( $A = \epsilon cl$ ); the path-length ( $l$ ) is a variable in this formula, thus it was necessary to calculate it for various volumes in the microplate. Figure 4.4.1.1. shows the relationship between the volume in the well and path-length for the microplates used throughout this thesis. The moles of carbonyl per mole of protein is assumed to be equivalent to the moles of DNPH per mole of protein, since one carbonyl group reacts with one



molecule of DNPH. The calculation of the moles of carbonyl per mole of protein would have been easier to determine using a calibration curve. I was, however, unable to think of an appropriate compound that would serve this assay as a calibrant. The calibrant would have to react with DNPH and be insoluble in TCA and ethanol/ethyl acetate; a protein is the obvious choice for the assay conditions, but native proteins do not exhibit enough moles of carbonyl to be accurately detected by this method. Glycated protein could have been used, but another assay would need to be used to determine its moles of carbonyl per mole of protein and again there is the question of calibration. If more time had been available for the development of the assay, I would have tried to tackle the problem of calibration more thoroughly.

The absorption spectra of fructated BSA after reaction with DNPH, along with its appropriate sample blank, is shown in figure 4.4.1.2. The wavelength of the maximum absorbance of the DNPH-protein adduct is 375nm. The nearest standard filter for the microplate reader was 379nm. A 375nm filter could, at extra cost, have been especially manufactured for us, but since the filters do not absorb the 10nm either side of their quoted peak, it was felt that the extra expense was not necessary.

Washing excess DNPH away from the protein-hydrazone was important because of its high absorbance at 379nm. Figure 4.4.1.3. shows that 4 washes with 1:1 ethanol:ethylacetate were sufficient to remove the vast majority of excess reagent. The solubility of BSA in ethanol:ethylacetate was a point of error reported by Levine *et al* (1990), thus the loss would need to be checked for each protein assayed [170]. I found, however, nearly 100% recovery and regarded any such loss with BSA as negligible.

Figure 4.4.1.4. shows the result of the DNPH assay on a sample of fructated BSA diluted into native BSA, such that the concentration of glycated protein is reduced, while the overall protein concentration is maintained. The undiluted sample ( $40\text{mg ml}^{-1}$ ) contained approximately 1 mole of carbonyl per mole of protein. The figure shows that if the fructated BSA is diluted into native BSA and assayed with DNPH, the absorbance of DNPH is directly proportional to the concentration of the protein down to  $0.25\text{mg ml}^{-1}$ . This means that, at a BSA concentration of  $10\text{mg ml}^{-1}$ , the DNPH assay can detect as little as 0.025 moles of carbonyl per mole of BSA. Below 0.025 moles of carbonyl per mole of protein the absolute absorbance of the DNPH-protein adduct is probably too small for an accurate calculation. The loss of accuracy at low concentrations of carbonyl groups is illustrated by the poor interassay coefficient of variation (CV) of the sugar-free control samples of figure 4.4.1.5.

Figure 4.4.1.5. illustrates two important points. The first is that it shows that the production of protein-bound carbonyl groups only occurs upon glycation. The sugar-free controls exhibit a slight increase in DNPH positive material, as a function of time, that is likely to be due to oxidation. The second point is that it shows that the CV of the fructated samples are all less than 2%, which is an acceptable, although undesirable, error between assays. The results of the sugar-free controls, however, resulted in a CV of more than 5%, which is probably due to exceeding the lower limit of the assay; this means that the results of the non-glycated samples can only indicate trends rather than absolute values of moles per mole.

Poor solubility of fructated BSA, after reaction with DNPH in 6M guanidine hydrochloride (pH 2.3), was an initial problem of the assay that was not resolved by heating. The solution to the problem was to freeze the sample at  $-20^{\circ}\text{C}$  and



subsequent thawing. Figure 4.4.1.6. shows the results from the same set of samples assayed with and without the final freezing step; there is quite a dramatic increase in yield with the freezing step. A possible explanation for this is that the freeze-thaw action on the protein pellet may increase the surface area available for the solvent to act upon and thus dramatically improve the solubility. All the DNPH assays used in this thesis included the freezing step, unless otherwise specified.

#### 4.5.2. Identity of periodate positive material from fructation

The production of periodate positive material (PPM) upon glucation or fructation of native or delipidated BSA is shown in figure 4.4.2.1. The amount of PPM produced from fructation is very much less than from glucation, because the aldehydic form of Heyns product does not yield formaldehyde on periodate oxidation. The minor ketone form of Heyns product will, however, yield formaldehyde upon periodate oxidation, thus the positive result seen on fructation could be from that moiety [209 and 111]. Examination of the production of PPM upon fructation (Fig. 4.4.2.1.), reveals a difference between native and delipidated BSA. The results suggest that PPM production upon fructation of BSA is, to a degree, dependent on the presence of lipid. In contrast, glucation of BSA is not affected by lipid. The higher initial level of Amadori product in glucated dBSA is possibly a reflection of the difference between the batches of BSA used to prepare the native and delipidated BSA. Some of the PPM observed upon fructation could be from Amadori product formed directly from glucose; glucose and fructose share a common enediol, thus one can be converted to the other. If, however, the PPM observed upon fructation were from glucose, it would not be inhibited by delipidation, thus the majority of PPM produced during fructation cannot have been formed using this route. The ketone form of Heyns product will produce PPM but if its production is analogous to formation of Amadori product, then it



should also be unaffected by the lipid component [8]. Some Amadori product can, in theory, be formed by rearrangement of Heyns product, and this could also be responsible for some of the PPM observed. Although this is possible the arguments above strongly suggest that the majority of PPM produced upon fructation is from unknown post-Heyns compounds.

#### 4.5.3. Identity of protein-bound carbonyl groups

Figure 4.4.2.2. shows that fructation produces protein-bound carbonyl groups at a faster rate than glucation, even though the glucose was at a concentration some 40 times higher than fructose. The graph also illustrates the necessity for lipid in production of protein-bound carbonyl groups, for both glucation and fructation. Similarly fructation produces a much higher fluorescence than glucation of BSA and the production of AGE-fluorophores is less in dBSA than nBSA (Fig. 4.4.2.3.).

Figures 4.4.2.2. and 4.4.2.3. indicate that the majority of production of protein-bound carbonyl groups and fluorescence requires the presence of the lipid component of BSA. There is, however, a slight increase in these measures upon glycation of dBSA, thus there must also exist lipid independent pathways to the production of protein-bound carbonyl and fluorescence. Further investigation of the effect of lipid on protein-bound carbonyl formation showed that fructation of nBSA for 10 days, and subsequent removal of the lipid, resulted in a 60% loss of carbonyl groups; this figure drops to 30% after a further 11 days fructation.

The data presented in this thesis indicate that protein-bound carbonyl groups only occur in the presence of lipid; and that the majority of the carbonyls detected by the DNPH assay occur initially on the lipid component and later, on the protein component of BSA. Comparing the build up, from glucation and fructation, of PPM, protein-bound carbonyl and fluorescence on dBSA, suggests; first, that the

effect of lipid on AGE formation is a post-Amadori or post-Heyns event; second, that the PPM observed is also from post-Heyns compounds; third, that the DNPH assay detects carbonyl groups formed on compounds produced from both Amadori and Heyns products.

Inhibition of AGE formation and a simultaneous build up of Amadori product has been reported by Fu *et al* (1994), for a variety of compounds that can therefore be assumed to affect post-Amadori or post-Heyns reactions [179]. Figure 4.4.3.1. shows the promotion of Amadori product formation by the metal chelator DTPA and the hydrazine compound aminoguanidine. The promotion of Amadori product, but inhibition of AGE-fluorescence, by DTPA and aminoguanidine, indicates that the compounds block the Maillard reaction at a point between the two measures of glycation (Figs. 4.4.3.3. and 4.4.3.5.).

DNPH should in theory react with the same compounds as aminoguanidine because they are both hydrazine compounds. Thus elucidation of the point in the Maillard reaction detected by DNPH, should give insight into the inhibitory action of aminoguanidine. The effect of aminoguanidine on production of PPM, protein-bound carbonyl groups and AGE-fluorescence (Figs. 4.4.3.1., 4.4.3.2. and 4.4.3.3.) gives support to the theory of DNPH and aminoguanidine competing for sites of action, i.e. promotion of Amadori product, complete inhibition of AGE-fluorescence and partial inhibition of protein-bound carbonyl formation. The partial inhibition could occur because the protein-aminoguanidine adduct is hydrolysed by the acid used to solvate DNPH reagent and thus regenerates the parent carbonyl compound [152]. Alternatively, aminoguanidine may have more than one site of action, before and after the point detected by DNPH (assuming that it detects a specific point in the Maillard reaction). A later point in the Maillard reaction, for which aminoguanidine has a higher affinity, would explain



partial inhibition of protein-bound carbonyl but complete inhibition of AGE-fluorescence.

The metal chelator DTPA also promotes Amadori product formation, while inhibiting both protein-bound carbonyl and AGE-fluorescence formation (Figs. 4.4.3.1., 4.4.3.4. and 4.4.3.5.). This indicates that DNPH detects protein-bound carbonyls produced as a result of post-Amadori or post-Heyns reactions and that these reactions are metal dependent.

The lipid and inhibitor experiments strongly indicated that DNPH is detecting a post-Amadori or post-Heyns intermediate of the Maillard reaction, thus an experiment was set up to confirm this by investigating post-Amadori or post-Heyns reactions without the influence of further glycation by free sugar. Figure 4.4.4.1., however, shows only a slight increase in formation of periodate positive material upon sugar-free re-incubation of fructated BSA. The increase was however very slight and when the Amadori product formed in the initial incubation (to produce fructated protein) was subtracted, the increase was the same in all of the fructated samples (lower part of figure 4.4.4.1.). This, along with the increase in periodate positive material of the sugar-free control, suggests that the increase during sugar-free re-incubation is due to oxidation rather than post-Amadori or post-Heyns reactions. Fructated protein does show a greater increase in PPM than the non-glycated protein which could be due to a conformational change in the protein, as a result of fructation producing a greater susceptibility to oxidation.

Figure 4.4.4.2. shows that post-Amadori or post-Heyns reactions lead to a decrease in protein-bound carbonyl groups that is proportional to the degree to which the BSA was initially fructated (as measured by days of fructation). This indicates that the protein-bound carbonyl detected by DNPH is an early or intermediate



compound in post-Amadori or post-Heyns reactions rather than an end-product. The protein-bound carbonyl groups, detected by DNPH and sequestered by aminoguanidine, may be precursors of the fluorescent AGEs. These conclusions are supported by the increase in fluorescence seen in the same samples during sugar-free re-incubation, where again the change is proportional to the degree of initial fructation (Fig. 4.4.4.3.).

Compounds that have been isolated from *in vitro* glycation mixtures and are possible candidates for reaction with DNPH and thus with aminoguanidine include: 1) the acyclic forms of Amadori product and Heyns product, although the results of this thesis would indicate that these are not major products detected by DNPH; 2) pyrrolaldehydes and their dimers, maltozazine (produced by reaction of 3-DOG with the amino acid proline, see page 48 *et sequa* for reactions of 3-DOG); 3) 3-DOG itself. Small free molecules (such as maltozazine and 3-DOG), however, would be soluble in TCA and ethanol/ethyl acetate and are thus not necessarily detected by this assay [212].

## **4.6. Summary**

- 1. This chapter describes an assay for glycation (including fructation) based on detection of protein-bound carbonyl groups by reaction with 2,4-dinitrophenylhydrazine.**
- 2. The production of protein-bound carbonyl groups and AGE-fluorescence are both dependent on the presence of lipid and free metal.**
- 3. The production of PPM upon fructation is dependent on lipid and thus the periodate assay is detecting a post-Heyns compound.**
- 4. The protein-bound carbonyl groups detected by DNPH are post-Amadori or post-Heyns intermediates in the Maillard reaction that occur before fluorophore formation.**
- 5. The principle sites of action, within the Maillard reaction, of the AGE inhibitor aminoguanidine are probably the same post-Amadori or post-Heyns intermediates detected by DNPH.**

## 5. Cross-Linking

### 5.1. Introduction

Advanced glycation end-products (AGEs) include intra- and intermolecular covalent cross-links, for example the fluorescent AGE biomarker pentosidine (Fig. 1.3.1.1.) is a cross-link between lysine and arginine residues [175]. As with other types of AGE produced *in vitro*, post-Amadori or post-Heyns reactions that lead to cross-linking are dependent on metal-catalysed oxidation (MCO) [176, 177 and 134].

Collagen is a major structural protein of the body that has a very long half life, which means that it can accumulate a large number of AGEs. This can, in turn, contribute to formation of secondary complications. *In vitro* glycation of collagen (from rat tail tendon) leads to loss of solubility, increased resistance to degradation (enzymatic and chemical) and accumulation of yellow and fluorescent pigments [178 and 179]. *In vitro* glycation of human tissues rich in collagen, such as the cornea or glomerular basement membrane (GBM), produces the same types of AGEs as those found in the rat tail collagen [177 and 180]. AGE cross-linking between collagen fibrils of the cornea disrupts their regular packing. *In vitro* glycated GBM traps more serum proteins, in the absence of free sugar, than native GBM. While it is implied that the serum proteins are covalently cross-linked to the GBM, this has not to my knowledge been conclusively proven [181]. A similar accumulation of LDL into glycated arterial wall collagen has been suggested as a contributory factor in vascular disease [182].

Similarly to *in vitro* glycation, *in vivo* glycation of collagen causes it to become less soluble, more cross-linked and results in accumulation of yellow and fluorescent



pigments; these changes occur as a function of age in normal individuals. The collagen-linked fluorescence of severe atherosclerotic lesions, is greater than that of the vessel wall of a person without macrovascular disease, indicating a greater concentration of AGEs in the former. Interestingly, collagen from superficial plaques (fatty streaks) exhibits a lower fluorescence than the non-diseased controls. Lee *et al* (1993) suggested that the lower fluorescence in superficial plaques was because this stage of the disease attracts the attention of macrophages. Macrophages induce intensive tissue remodelling, which probably masks AGE formation by replacement of the collagen [187 and 188]. Basement membranes thicken as a function of age and this is accelerated in diabetes, although whether this is due to cross-linking of the collagen in the membrane or some other process is not clear [189].

Although the changes in collagen-rich tissues, described in the paragraph above, increase as a function of age, people with diabetes mellitus show the same changes but at an earlier age, in this respect diabetes is an ageing disease [187]. People with diabetes mellitus also show increased incorporation of serum proteins into structural collagen elements such as basement membranes. There is *in vivo* evidence that diabetics have a greater amount of IgG and IgM incorporated in peripheral nerve myelin than age matched controls [190]. The concentration of trapped HSA and IgG in renal basement membranes is also higher in diabetic kidney as opposed to non-diabetic kidney with other renal diseases, and normal controls (free of any disease) [191]. Increased deposition of albumin has also been reported in extracellular dermal capillary basement membrane in patients with IDDM, compared to non-diabetics [192]. Such incorporation of serum proteins into structural elements of the body is thought to exacerbate vascular pathogenesis and renal disease; thus the increased levels in diabetes would explain the earlier onset

of these complications in diabetics than in people of the same age without the disease [182 and 191].

Deposition of amyloid fibrils on the synovial membrane of joints is a serious complication of patients on long term haemodialysis. Analysis of the fibrils indicates that glycated  $\beta_2$ -microglobulin is a major component. *In vivo* glycated  $\beta_2$ -microglobulin, isolated from urine of diabetics, enhances *in vitro* chemotaxis and chemokinesis of human monocytes. The macrophages release enough tumor necrosis

factor- $\alpha$  and interleukin- $1\beta$  to stimulate collagenase release from cultured synovial cells. These same cytokines are known to induce tissue re-modelling in a variety of tissues. Miyata *et al* (1994) proposed that an immune response to glycated  $\beta_2$ -microglobulin in amyloid fibrils could initiate inflammatory disease [193].

The breakdown products of enzymatic tissue remodelling of AGE-proteins may also cause problems *in vivo*. Makita *et al* (1994) found elevated concentrations of 2-6kD protein fragments in the serum of diabetics. Isolation of this molecular weight range revealed that the fragments had a high cross-linking potential with collagen. In people with normal kidney function the protein fragments have a very short half life in circulation, however their high reactivity suggests a possible role for exacerbation of vascular disease. The 2-6kD protein fragments are not, however, cleared in end-stage renal disease either by the kidney or the normal dialysis systems used to keep these patients alive. The resultant build up of the fragments in these patients probably contributes to the deterioration of their health [194].

AGE formation on collagen is an important area of research because of its ubiquity. Alteration of the properties of tissues containing collagen is probably responsible for many degenerative diseases. Collagen is not, however, the only protein to have AGEs form upon it. The *in vivo* cross-linking of blood proteins to



each other also probably contributes to diabetic secondary complications. Up to 21% of intraplatelet calmodulin was found to be glycated in a survey of diabetics, and hyperaggregation of platelets has been attributed to perpetuation of diabetic arteriosclerosis, hypertension and retinopathy [195]. Lubec *et al* (1993) reported that Maillard cross-linking of IgG is greater in diabetics than non-diabetics, but that such cross-linking was inhibited by oral administration of L-arginine. The group proposed that the L-arginine reacted with Amadori product and prevented its further part in the Maillard reaction [196].

*In vitro* studies of Maillard cross-linking are usually carried out to either show that a particular protein cross-links, or to investigate the mechanism of cross-link formation. The molecular weight of collagen makes it a difficult protein to study cross-linking in. Studies of proteins, other than collagen, that have been shown to cross-link *in vitro* include; IgG, lens proteins, RNase and lysozyme [183, 184, 185 and 186]. A principle aim of this thesis was to study AGE formation from fructose, including cross-linking and to relate the conclusions of this thesis to fructation *in vivo*. Fructose has been shown, *in vitro*, to cross-link collagen, BSA, lysozyme, and RNase at a faster rate than glucose [198, 199 and 200]. I chose to use lysozyme and  $\beta$ -lactoglobulin to study cross-linking because they have useful molecular weights for SDS-PAGE, also they are relatively inexpensive. There are a few problems with using these proteins that should be pointed out before the reader analyses the results. Lysozyme is contaminated with a number of proteins: several of lower molecular weight than lysozyme (i.e. <14kD), also ovalbumin (45kD) and an unknown protein of 73kD; each of which could confuse interpretation of the results.  $\beta$ -lactoglobulin has been purified from bovine milk which contains lactose at an average concentration of 0.13M [207]. This means that in any preparation of  $\beta$ -lactoglobulin there will be some glycated protein. The amount of glycated  $\beta$ -lactoglobulin, and similarly the AGEs, present in a preparation of the protein



depends on the duration of storage of the milk; such information is, unfortunately, not available from Sigma. The presence of some *in vivo* glycated  $\beta$ -lactoglobulin in the protein preparation can be confirmed by the presence of a  $\beta$ -lactoglobulin homodimer in the sugar-free controls, again this can confuse analysis of the results if it is not noted.

Knowledge of the mechanism of the formation of Maillard-cross-links would make possible the rational design of drugs to block the reaction *in vivo*, thus preventing the progression of diabetic secondary complications. Inhibition of Maillard cross-linking can be achieved *in vitro* by non-oxidative conditions or by sequestration of reactive groups. A useful review of some of the compounds that inhibit AGE formation can be found in Fu *et al* (1994) [134, 197 and 179]. Since the Maillard reaction produces compounds by metal catalysed oxidation, chelation of that metal can produce non-oxidative conditions. L-arginine (as mentioned above) and aminoguanidine are examples of compounds that will sequester reactive groups and block the Maillard reaction. The site of the inhibitory action of aminoguanidine is contentious, it is a hydrazine that will react with carbonyl groups presented at many stages in the Maillard reaction. For a more detailed discussion of aminoguanidine see pages 111 *et seq.*

## **5.2. Aims**

- 1. To illustrate Maillard-cross-linking upon fructation.**
- 2. To show that two different proteins can be covalently joined by Maillard-cross-linking.**
- 3. To study the effect of inhibitors of AGE formation on Maillard-cross-linking.**
- 4. To investigate the effect of incubating fructated and native proteins together in the absence of free sugar.**
- 5. To illustrate how low levels of fructation can still produce detectable AGEs in the form of cross-links.**

### 5.3. Methods

*In vitro* glycation was carried out according to the methods described on page 56 *et sequa*.

Precipitation by formation of high molecular weight aggregates was a problem in some experiments. If, for example, lysozyme ( $10\text{mg ml}^{-1}$ ) was glycated for 9 or more days with 0.5M fructose, or fructation (0.1M fructose) of lysozyme together with  $\beta$ -lactoglobulin ( $10\text{mg ml}^{-1}$ ) for 2 to 5 days, then precipitation occurred (results not shown). The samples containing precipitate were centrifuged (3 minutes, 14000g) and the supernatant diluted to an appropriate concentration for assay.

The cross-linking of fructated lysozyme ( $10\text{mg ml}^{-1}$ ),  $\beta$ -lactoglobulin ( $10\text{mg ml}^{-1}$ ) and BSA ( $40\text{mg/ml}$ ) was detected using SDS-PAGE, quantified by laser densitometry and compared to fluorescent AGEs (see pages 62 *et sequa*).

SDS-PAGE of denatured (boiled with SDS and 2-mercaptoethanol) fructated lysozyme,  $\beta$ -lactoglobulin and BSA was used to illustrate AGEs in the form of covalent, non-thiol, cross-links. Densitometry was used to quantify cross-linking as a percentage of the total staining in that lane and assumed that the intensities are such that absorbance is linear with concentration, according to the Beer-Lambert law. To further quantify the amount of protein that has become cross-linked, it was also necessary to assume that the cross-linked protein stained to the same degree as the native protein. Both of these assumptions have faults; the actual concentration of cross-linked protein is low compared to that of the native monomer so a certain degree of overloading is necessary to detect the cross-linking. Glycation clearly inhibits silver staining (Fig. 2.9.1.1.), thus such staining



cannot be relied upon for accurate quantification and so CCBB staining was preferred. CCBB staining was also preferred because the laser used in the densitometer emits light at the red end of the spectrum which is where the Coomassie brilliant blue  $\lambda_{\text{max}}$  occurs, whereas silver stain absorbs in the blue green region.

**Inhibition of cross-linking** was investigated by adding aminoguanidine (50mM) or the metal chelator diethylenetriaminepenta-acetic acid (DTPA, 1mM) to the phosphate buffer used during glycation. To further investigate the role of metal catalysed oxidation (MCO) and free radicals, the buffer HEPES (10mM), which is low in free metal and has free radical scavenging properties, was used as a substitute for phosphate buffer.

**Post-Heyns reactions** were investigated without interference from free sugar, by removal of excess free sugar from fructated protein, either by exhaustive dialysis or size exclusion chromatography (section 2.4.). A second non-glycated, native protein was added to the fructated protein and the two were re-incubated for 1 week at 37°C in either phosphate or bicarbonate buffer.

## 5.4. Results

### 5.4.1. Fructation-Induced Cross-linking

The production of AGE cross-links and fluorescence upon fructation of lysozyme is proportional to the period of glycation; figure 5.4.1.1. shows the production of a 28kD dimer and 42kD trimer. Densitometry of this gel (Fig. 5.4.1.2.) illustrates that the rate of oligomerisation is not linear with respect to the period of fructation. Formation of dimer ceases after 4 days, probably because the dimer is a precursor for another AGE, only produced when a critical amount of its substrates are present. The trimer is produced in lower yields than the dimer but at an almost continuous rate throughout the experiment. The top curve in figure 5.4.1.2., however, indicates that the rate of formation of dimer and trimer, considered together, is slowing. It is possible that two dimers are cross-linking to form a 54kD protein made up of four monomers; the gel in figure 5.4.1.1. does show darkening in lanes E and F (6 and 8 days fructation) in the 45 to 66kD region of the gel; the bands are, however, too faint and broad to positively confirm the production of a 54kD lysozyme oligomer. Precipitation was not a problem with this experiment, because the glycation of lysozyme at this concentration of fructose was for less than 9 days.

Figure 5.4.1.3. illustrates the pentosidine-linked fluorescence of the same samples of fructated lysozyme used in the gel of figure 5.4.1.1. After 8 days incubation the sugar-free control shows very little change in its fluorescence, while the fructated lysozyme has produced fluorophores at a rate that was initially large but is starting to form a plateau. It is conceivable that the fluorophores themselves, or intermediates in their production, are at the later stages of the Maillard reaction used to produce other AGEs.

Similarly fructation induces cross-linking of  $\beta$ -lactoglobulin. Figure 5.4.1.4. illustrates production of a 36kD dimer and a 54kD trimer of  $\beta$ -lactoglobulin upon 15 days fructation. The densitometry of this gel (Fig. 5.4.1.5.) shows that dimerisation ceases around day 10 while formation of trimer is linear throughout the duration of the experiment. The gel also shows an increase in staining, as a function of fructation, throughout the molecular weight range from 36kD upwards, with only the trimer at 54kD forming a discernible band, suggesting production of multimers of various molecular weights. Interestingly  $\beta$ -lactoglobulin fructated on its own did not precipitate even after 15 days incubation with 0.1M fructose. When  $\beta$ -lactoglobulin is incubated with lysozyme, however, precipitation occurs very quickly (2 to 5 days in 0.1M fructose). Figure 5.4.1.6. illustrates such high molecular weight aggregates produced after 7 days fructation (lane D). The figure also shows that fructation of  $\beta$ -lactoglobulin and lysozyme together (lane D) produces a 32kD heterodimer as well as the homodimers of the two proteins (32kD and 28kD respectively).

Oligomerisation can also be demonstrated in larger proteins. Figure 5.4.1.7. shows that when BSA is incubated for up to 7 days with 50mM fructose a new protein band is produced at 72kD. This shows a further increase in staining intensity, and thus in protein concentration, after 14 and 21 days of fructation. Faint protein bands also appear at 29kD and 33kD and there is an apparent loss of staining (thus a loss of protein) in the bands corresponding to BSA and other smaller contaminating globulins, as a function of the time of fructation.



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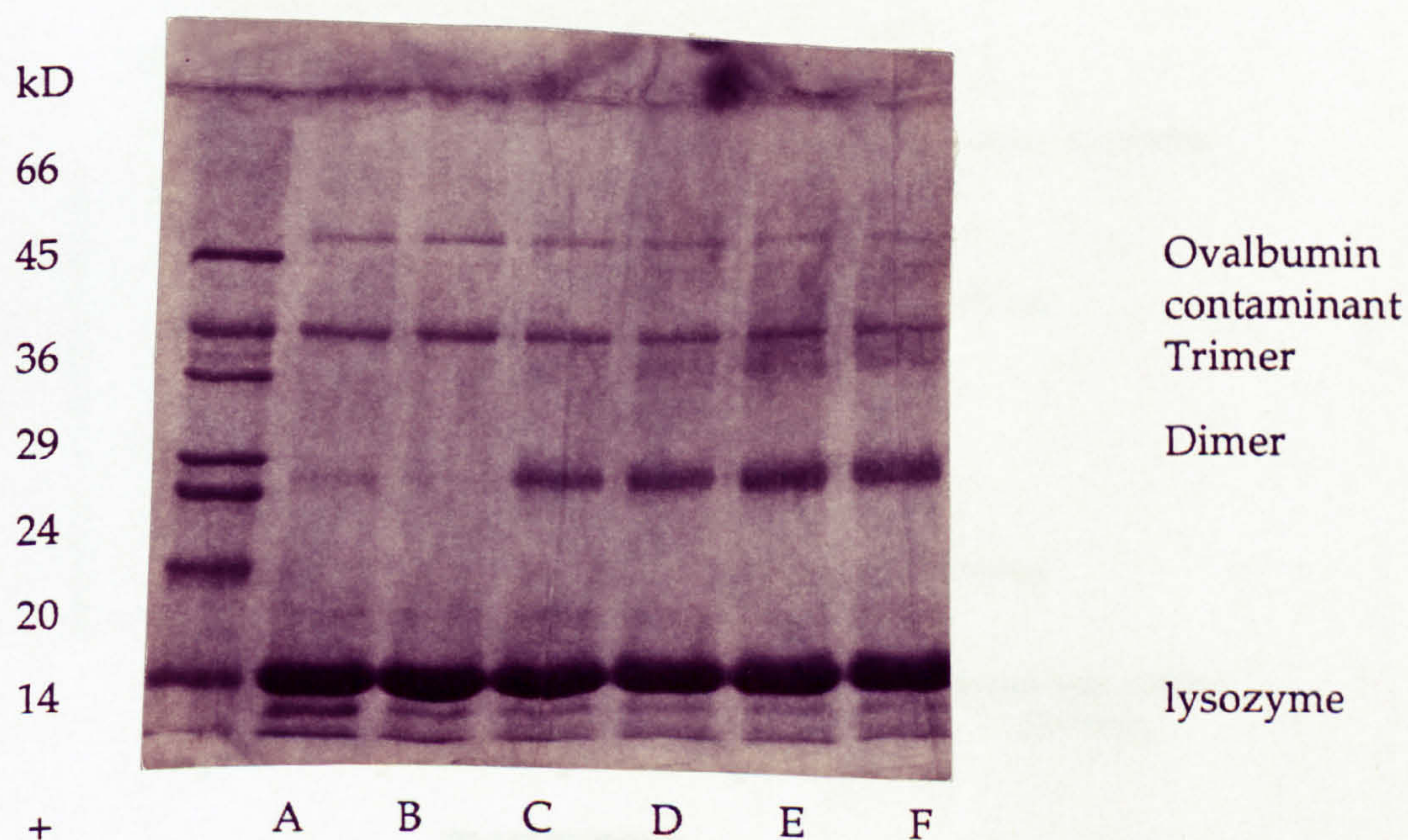


Fig. 5.4.1.1. Glycation (0.5M fructose) induced cross-linking of lysozyme produces a 28kD homodimer and a 42kD trimer. Lane A represents 8 days incubation in the absence of free sugar while lanes B, C, D, E and F represent 0, 2, 4, 6 and 8 days incubation with 0.5M fructose respectively. Ovalbumin is a contaminant of the lysozyme preparation.



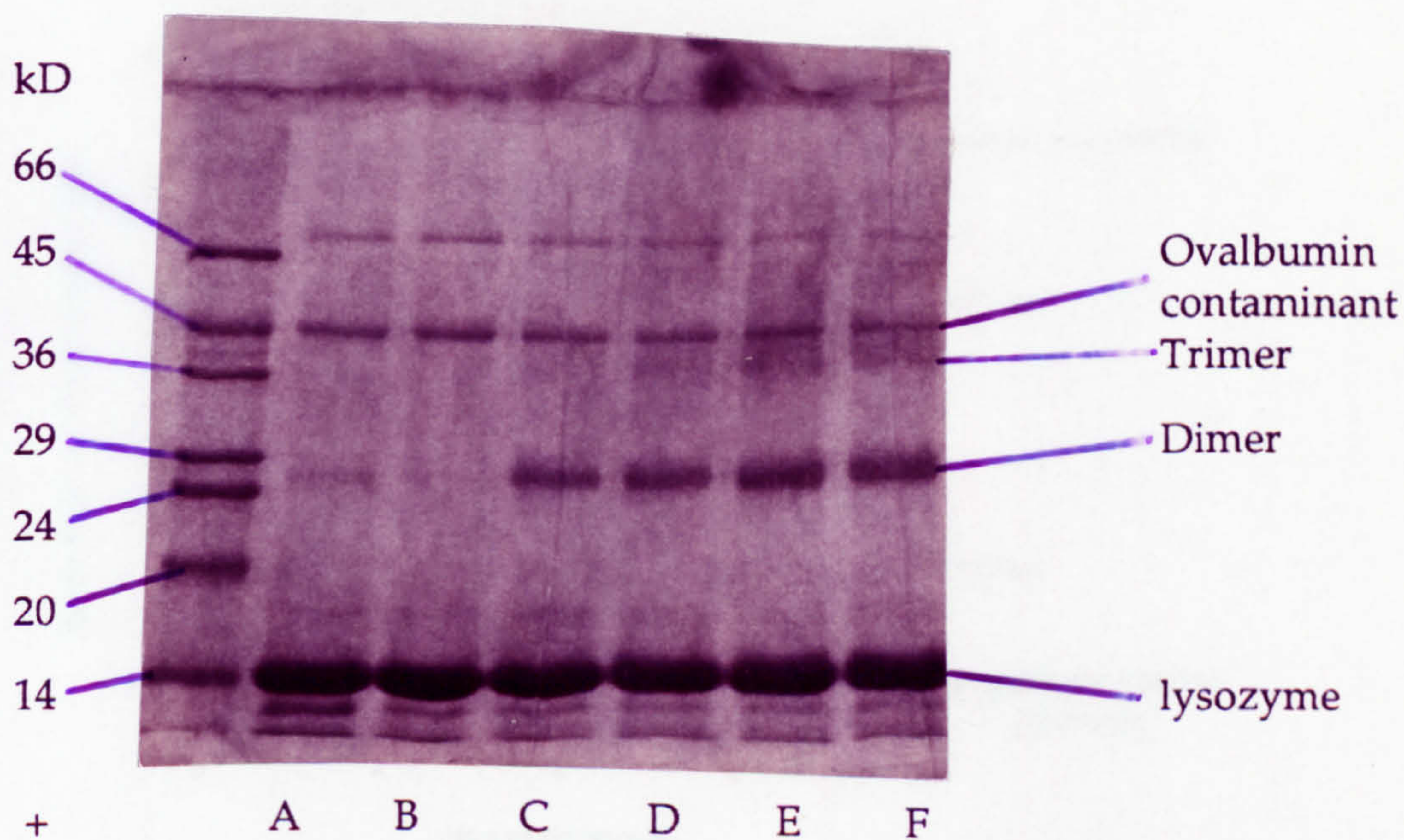


Fig. 5.4.1.1. Glycation (0.5M fructose) induced cross-linking of lysozyme produces a 28kD homodimer and a 42kD trimer. Lane A represents 8 days incubation in the absence of free sugar while lanes B, C, D, E and F represent 0, 2, 4, 6 and 8 days incubation with 0.5M fructose respectively. Ovalbumin is a contaminant of the lysozyme preparation.



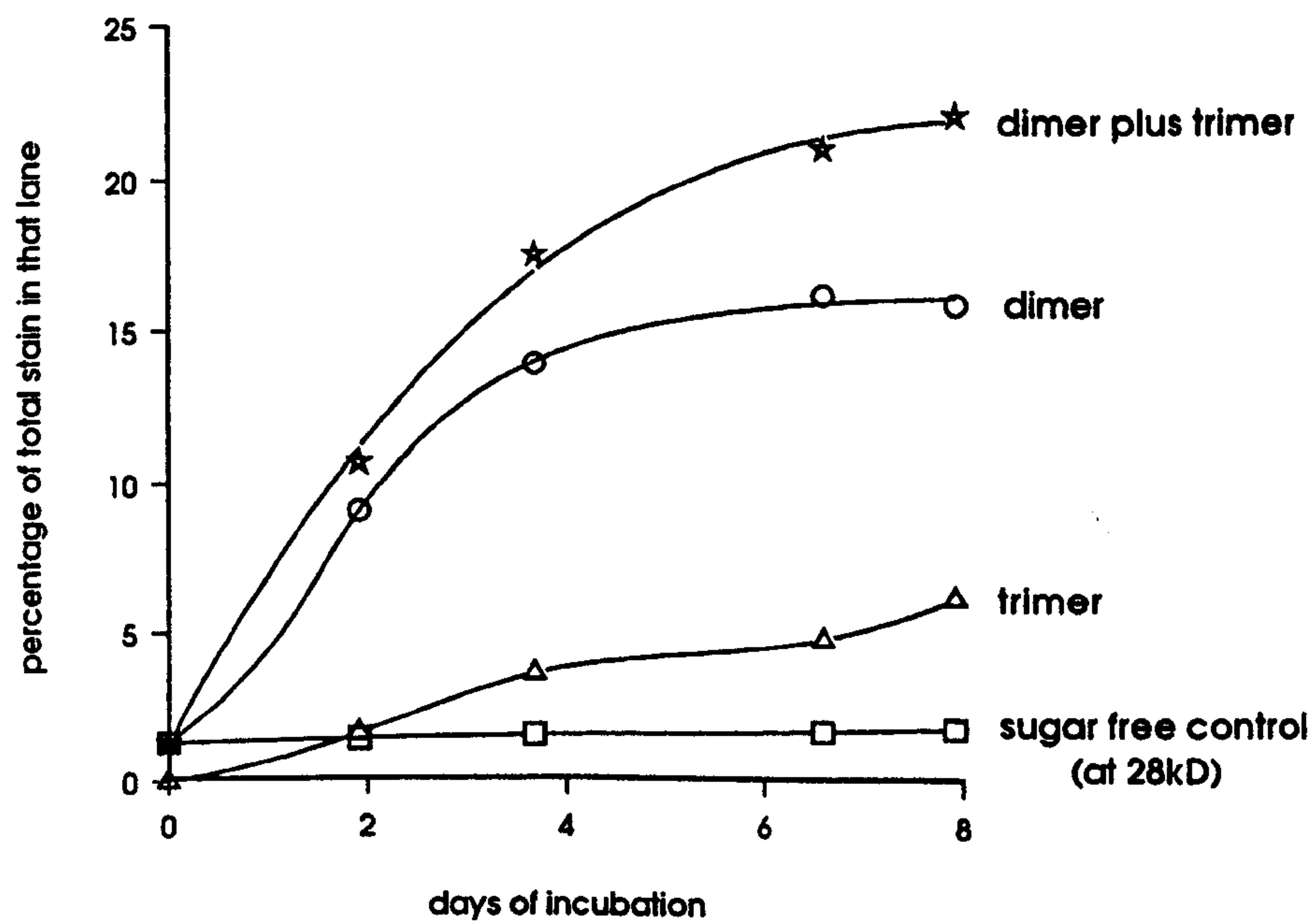


Fig. 5.4.1.2. Quantitation of fructation (0.5M fructose) induced cross-linking of lysozyme; samples taken from the gel depicted in figure 5.4.1.1.

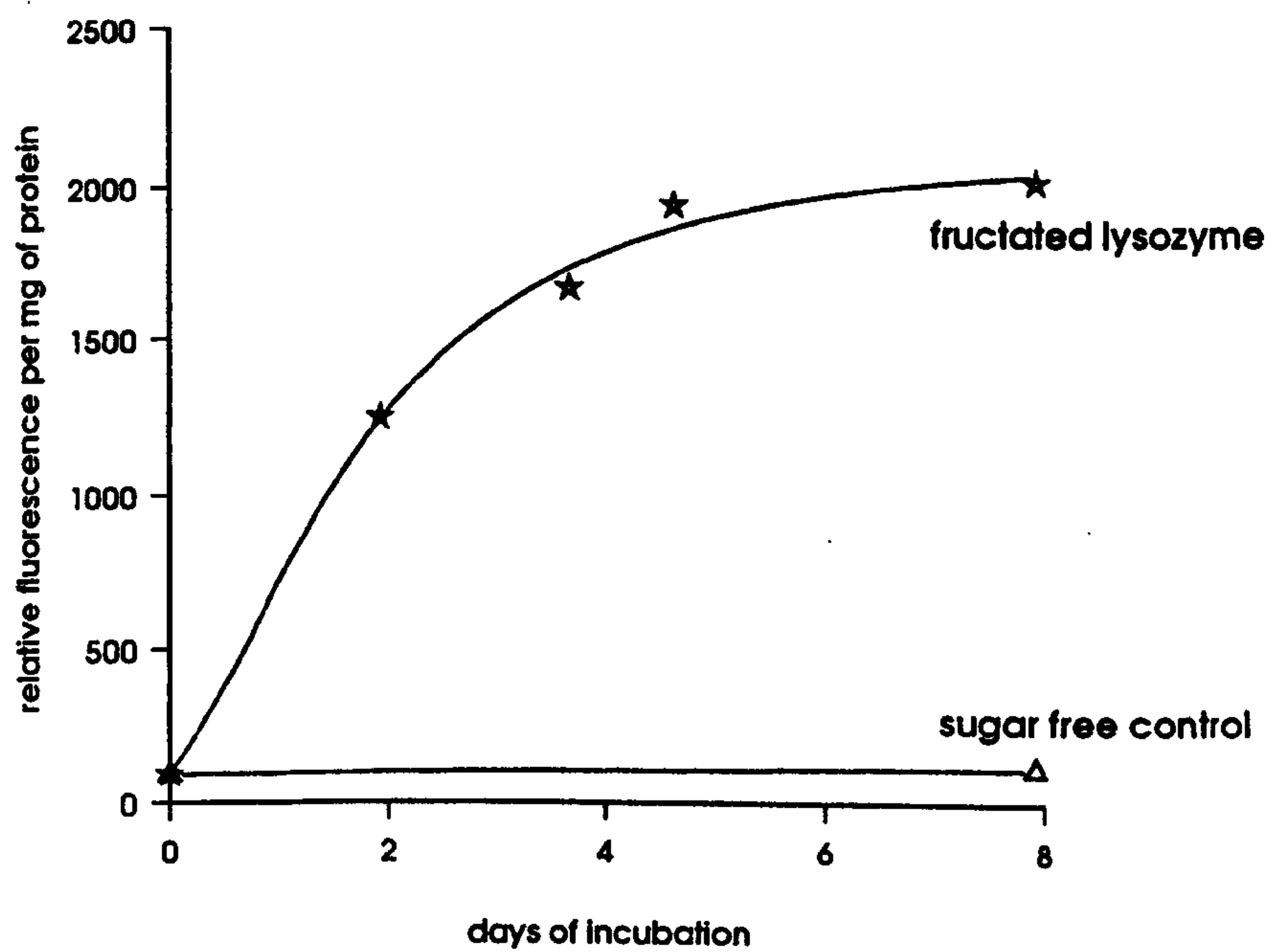
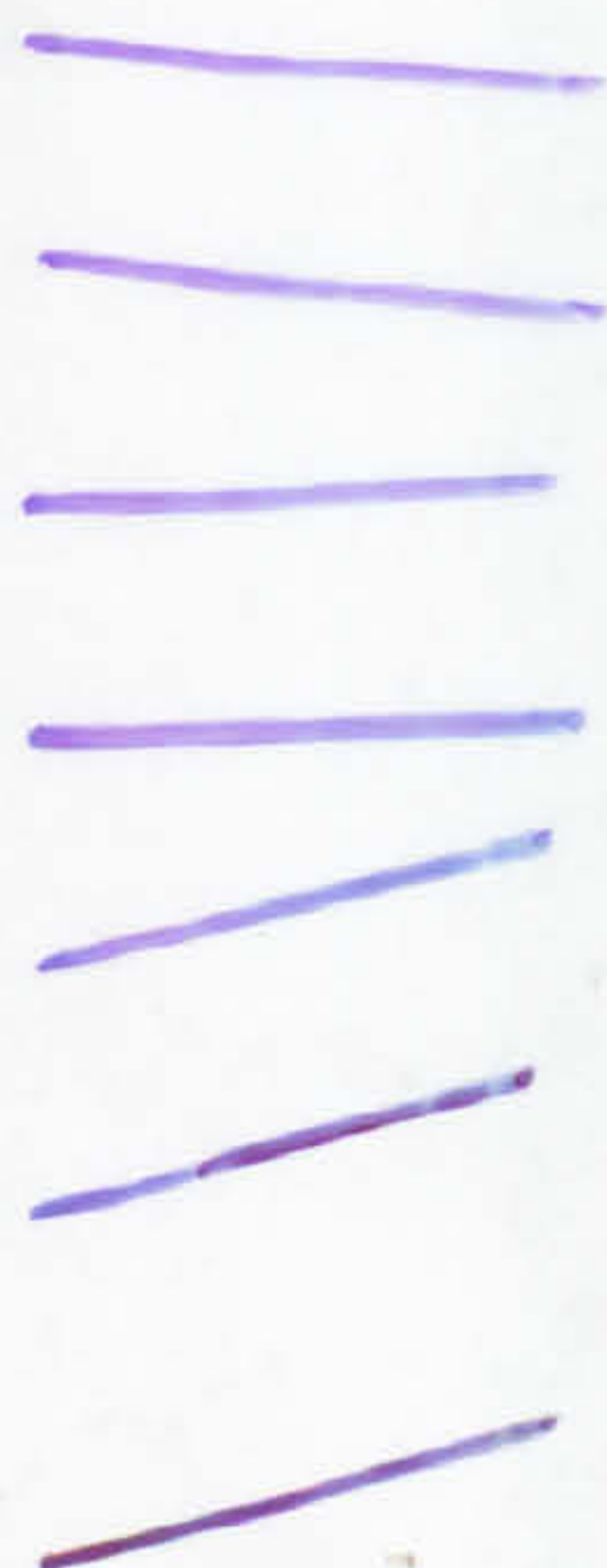


Fig. 5.4.1.3. Pentosidine-linked fluorescence of the fructated (0.5M fructose) lysozyme samples depicted in figure 5.4.1.1.





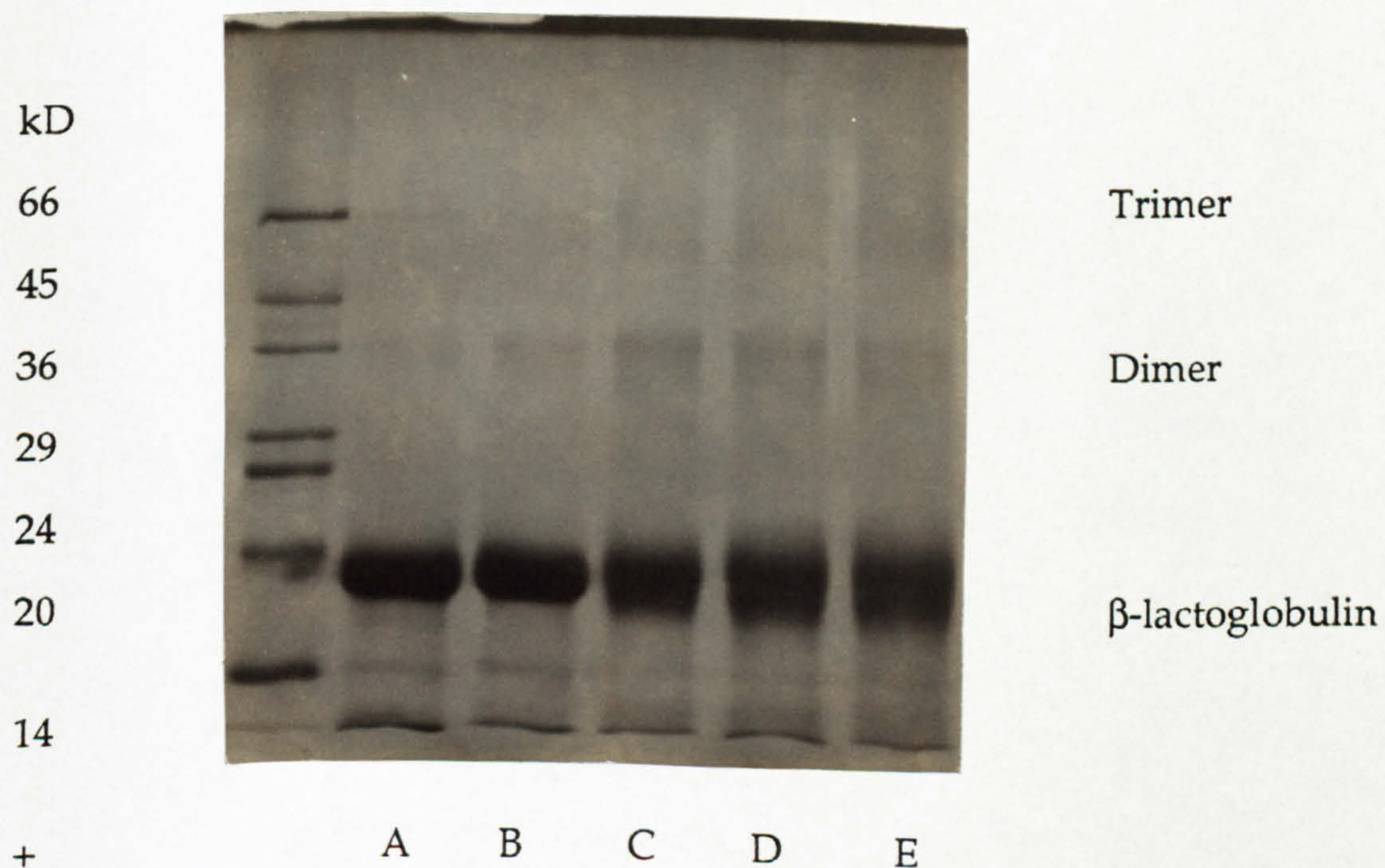


Fig 5.4.1.4. Glycation (0.05M fructose) induced cross-linking of  $\beta$ -lactoglobulin produces a 36kD homodimer and a 54kD trimer. Lane A represents 15 days incubation in the absence of free sugar while lanes B, C, D and E represent 0, 7, 11 and 15 days incubation with 0.05M fructose respectively.



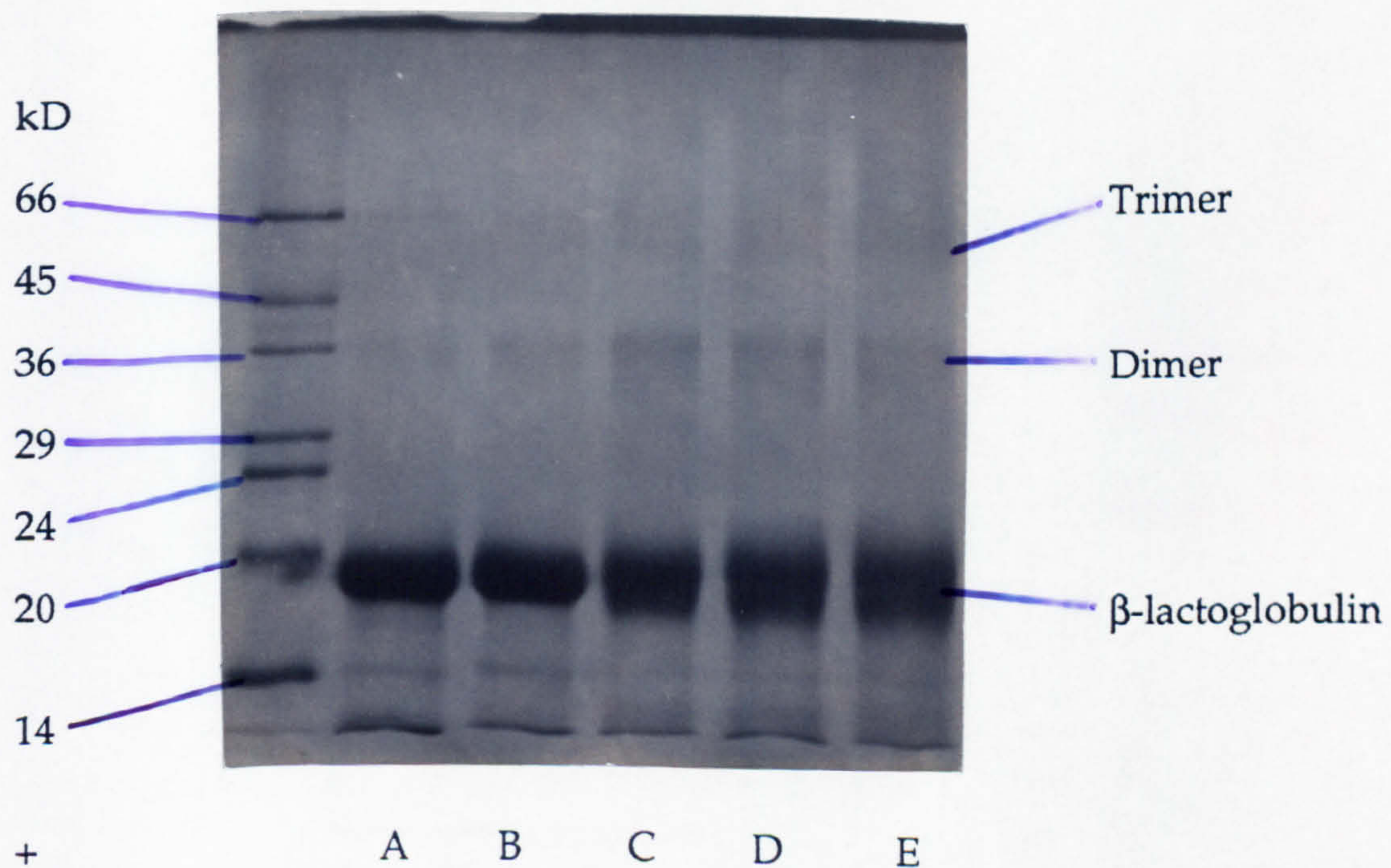


Fig 5.4.1.4. Glycation (0.05M fructose) induced cross-linking of  $\beta$ -lactoglobulin produces a 36kD homodimer and a 54kD trimer. Lane A represents 15 days incubation in the absence of free sugar while lanes B, C, D and E represent 0, 7, 11 and 15 days incubation with 0.05M fructose respectively.



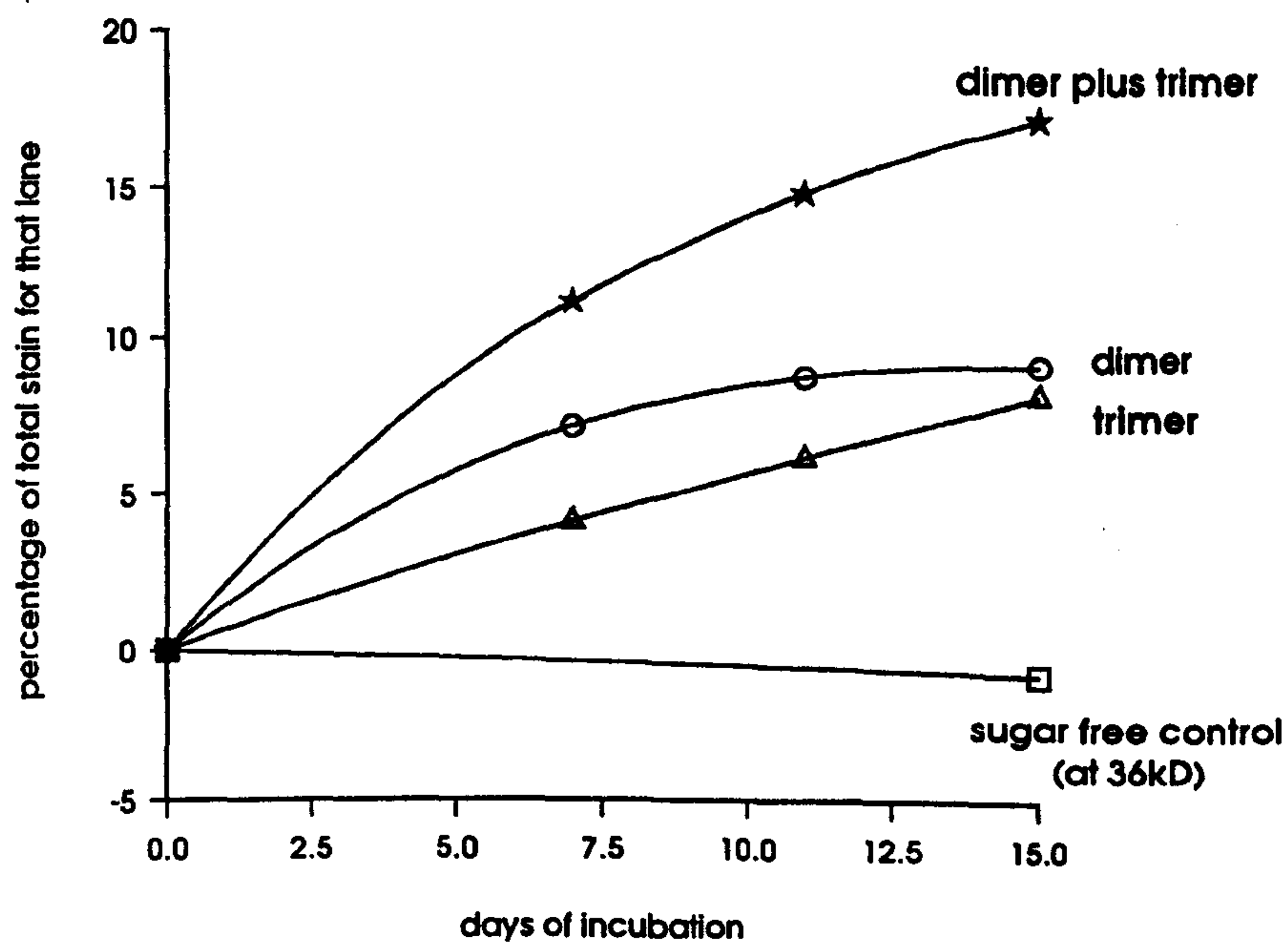
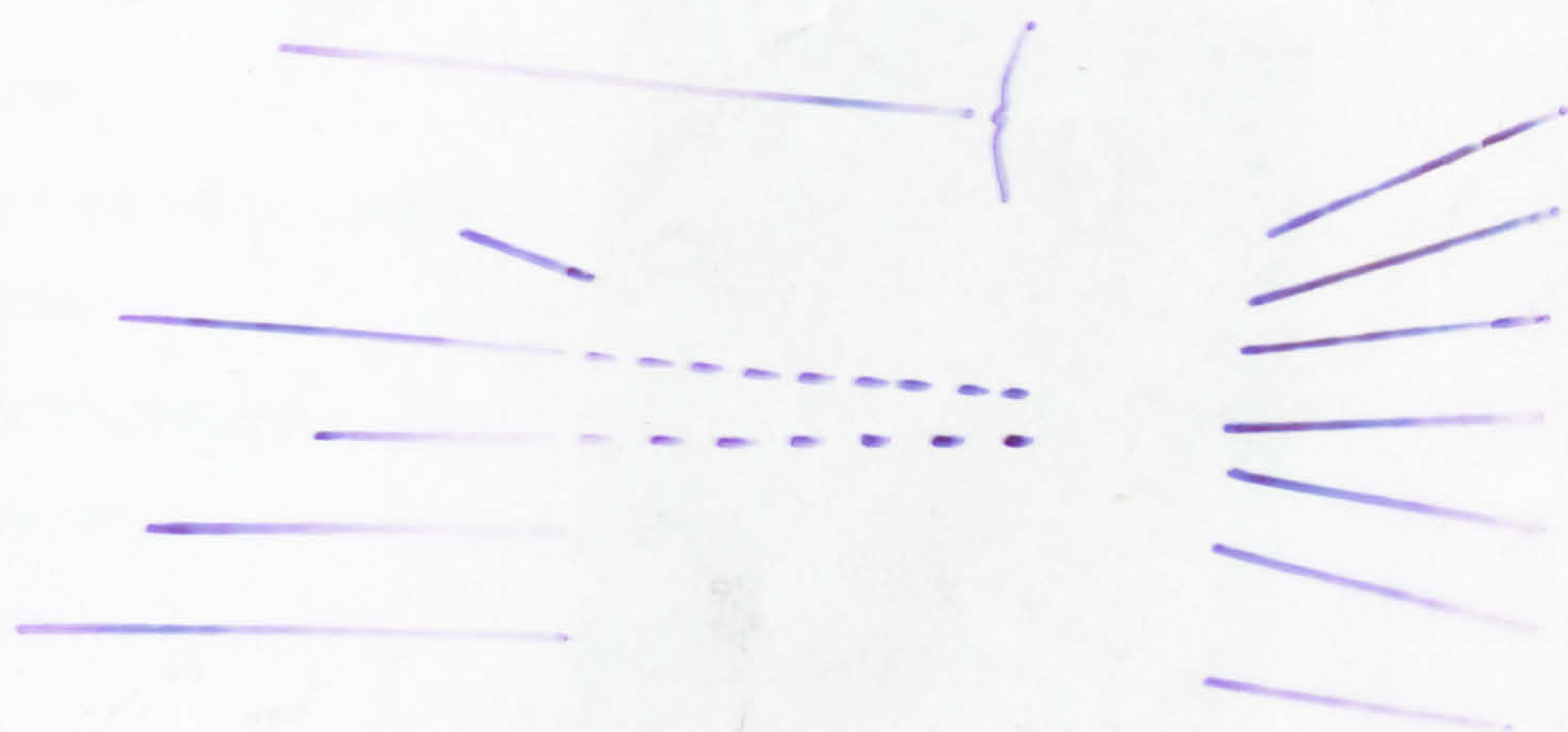


Fig. 5.4.1.5. Quantitation of fructation (0.05M fructose) induced cross-linking of  $\beta$ -lactoglobulin by densitometry of the gel depicted in Figure 5.4.1.4. The results presented are net, the dimer and trimer formed *in vivo* (lane B) has been subtracted from those formed *in vitro* (lanes C to E).







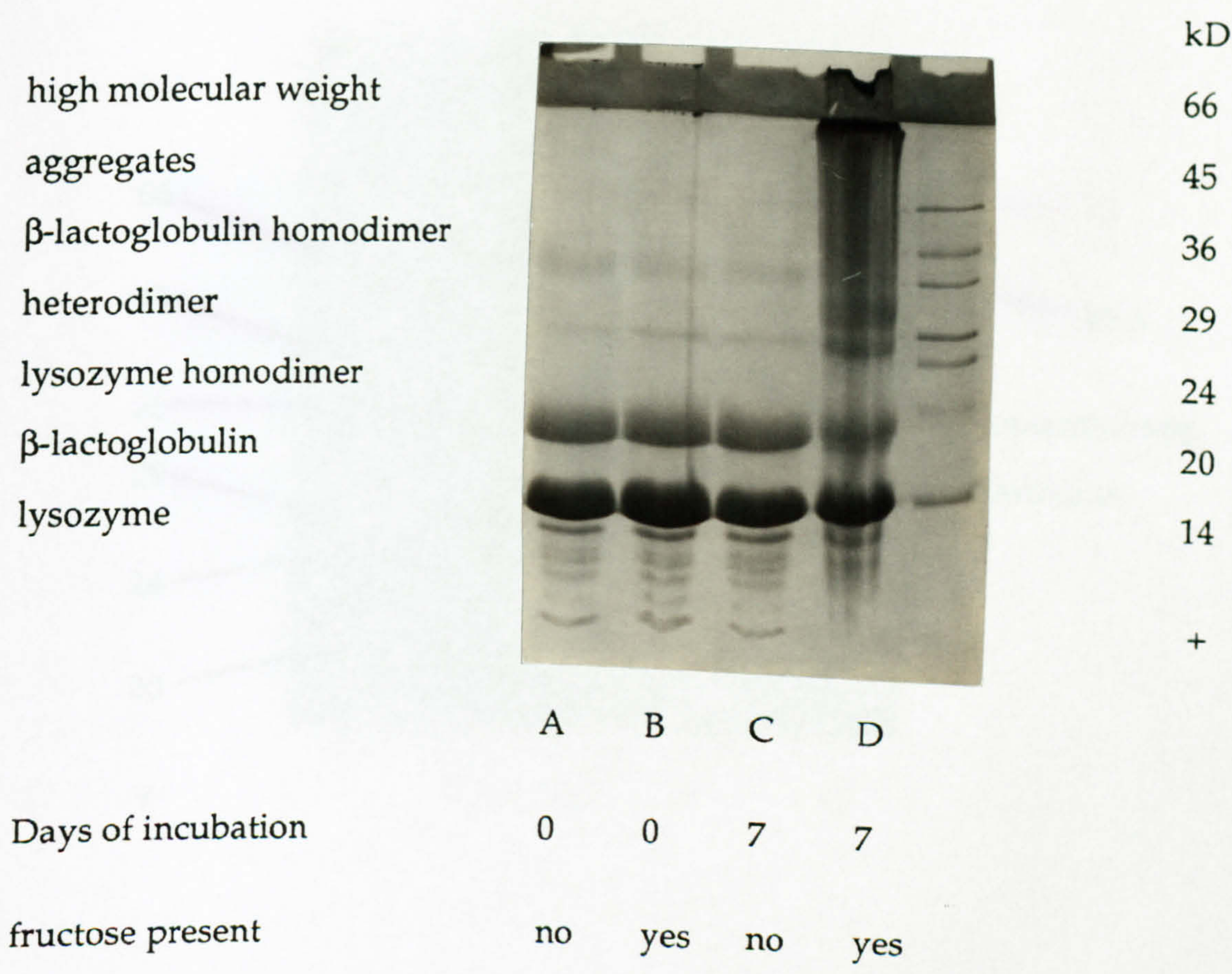


Fig 5.4.1.6. Glycation (0.1M fructose) of lysozyme and  $\beta$ -lactoglobulin together produces a 32kD heterodimer and high molecular weight aggregates. Lanes A and B show the protein before glycation; A contains no free-sugar while B contains 0.1M fructose. Lanes C and D show the same samples (A and B respectively) after 7 days incubation at 37°C.



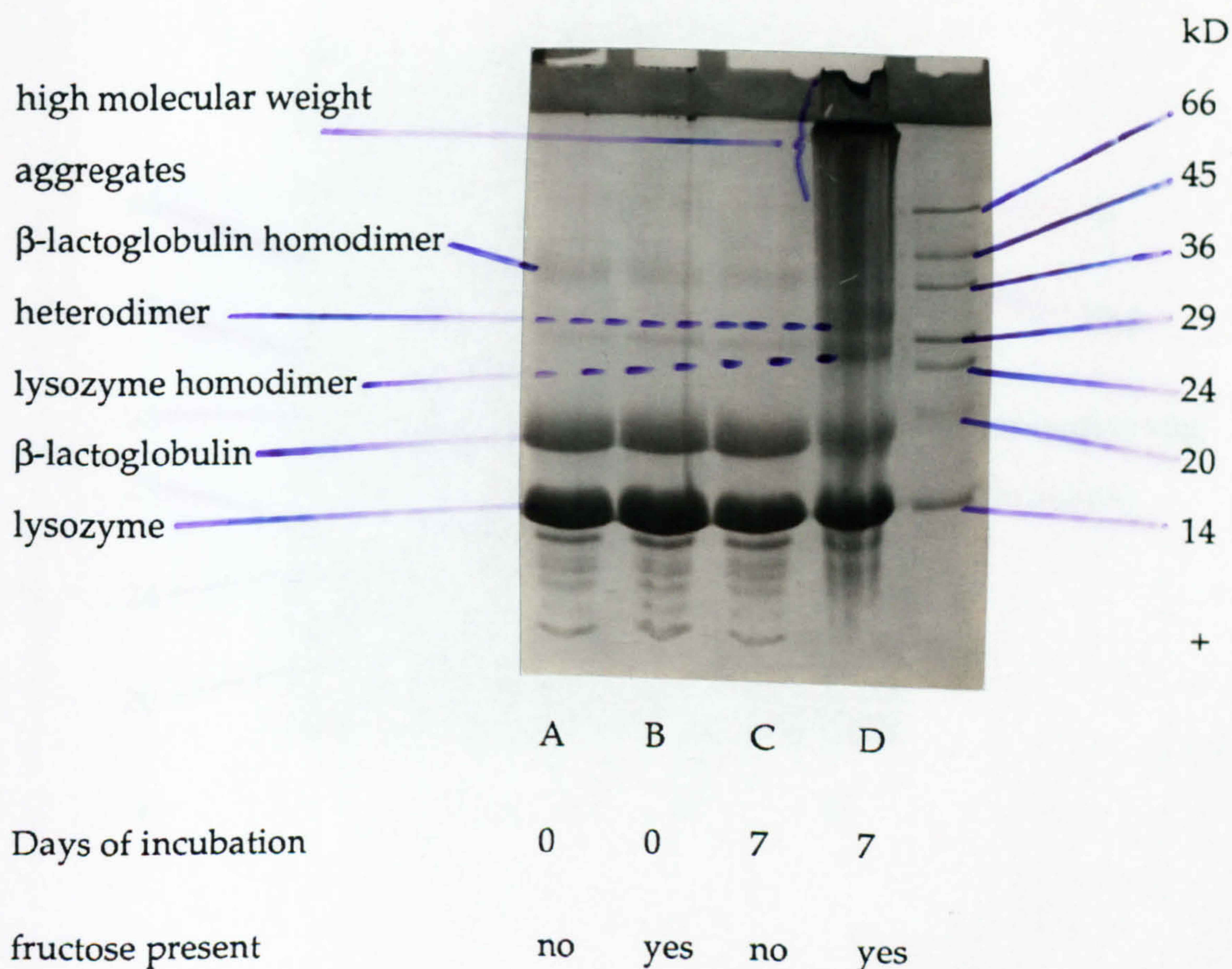
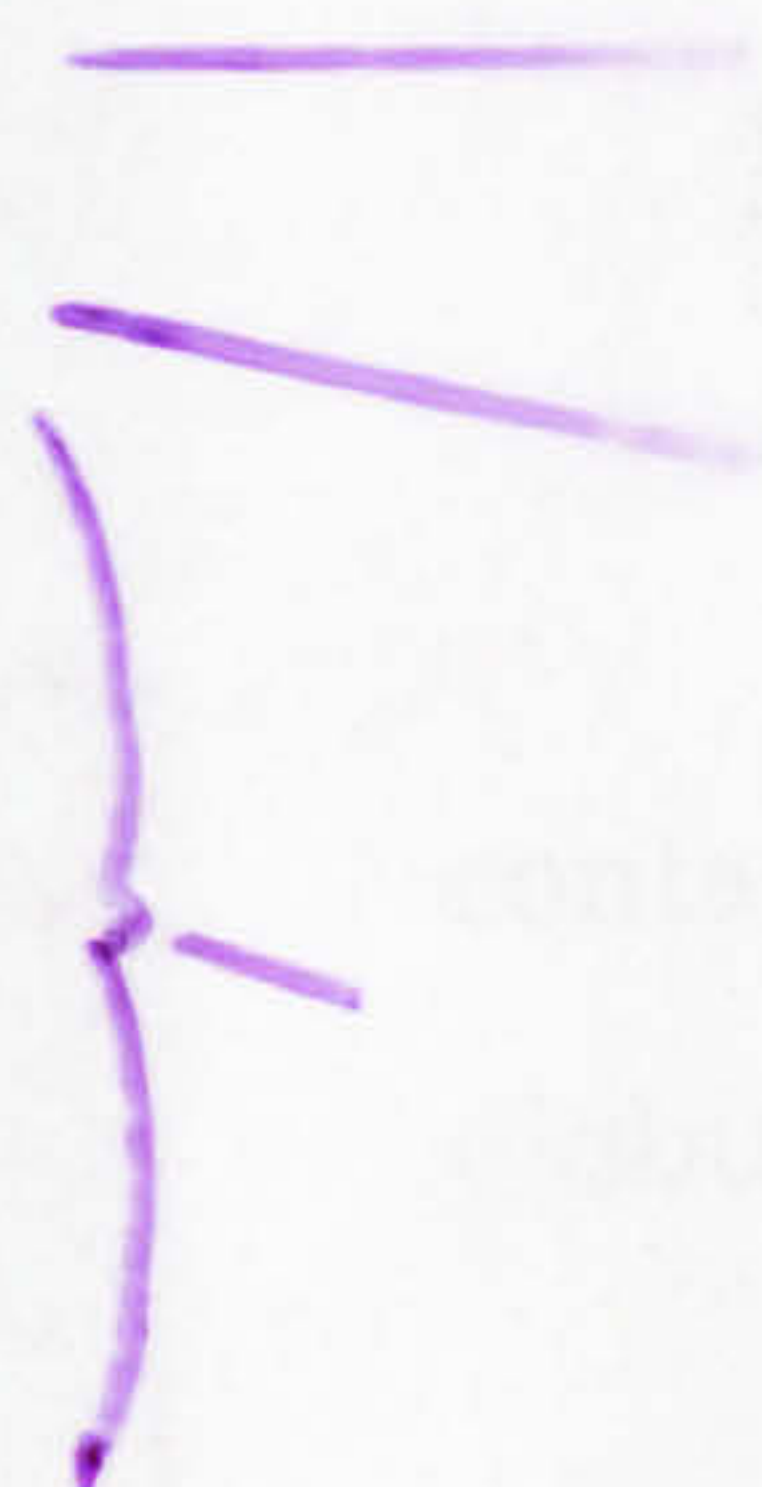


Fig 5.4.1.6. Glycation (0.1M fructose) of lysozyme and  $\beta$ -lactoglobulin together produces a 32kD heterodimer and high molecular weight aggregates. Lanes A and B show the protein before glycation; A contains no free-sugar while B contains 0.1M fructose. Lanes C and D show the same samples (A and B respectively) after 7 days incubation at 37°C.







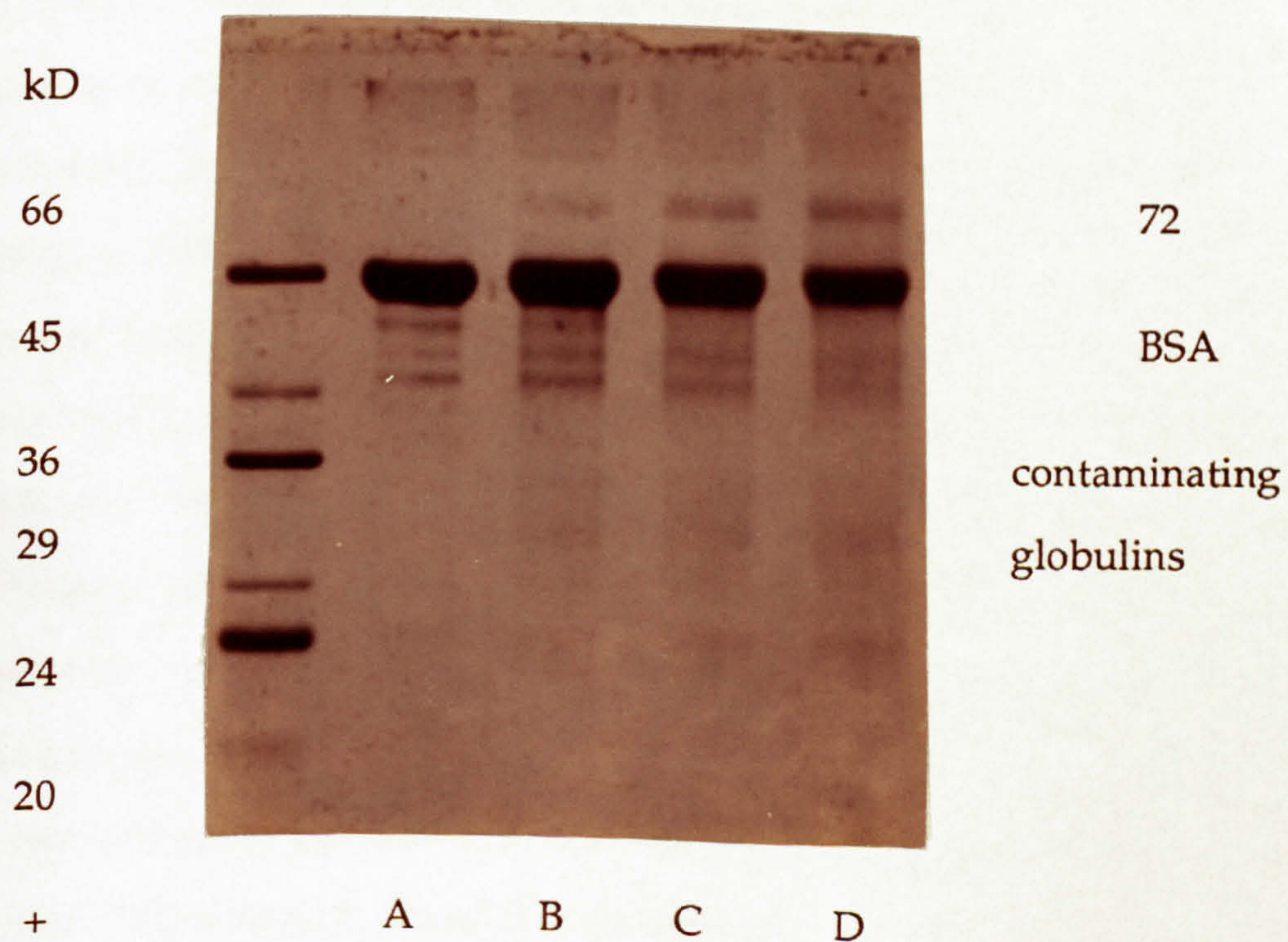


Fig 5.4.1.7. Fructation of BSA (fraction V) produces a 72kD protein band after 7 days incubation in 0.05M fructose. Lanes A, B, C and D represent 0, 7, 14 and 21 days fructation respectively. This gel was stained with Bio-Rad silver stain plus (page 65).



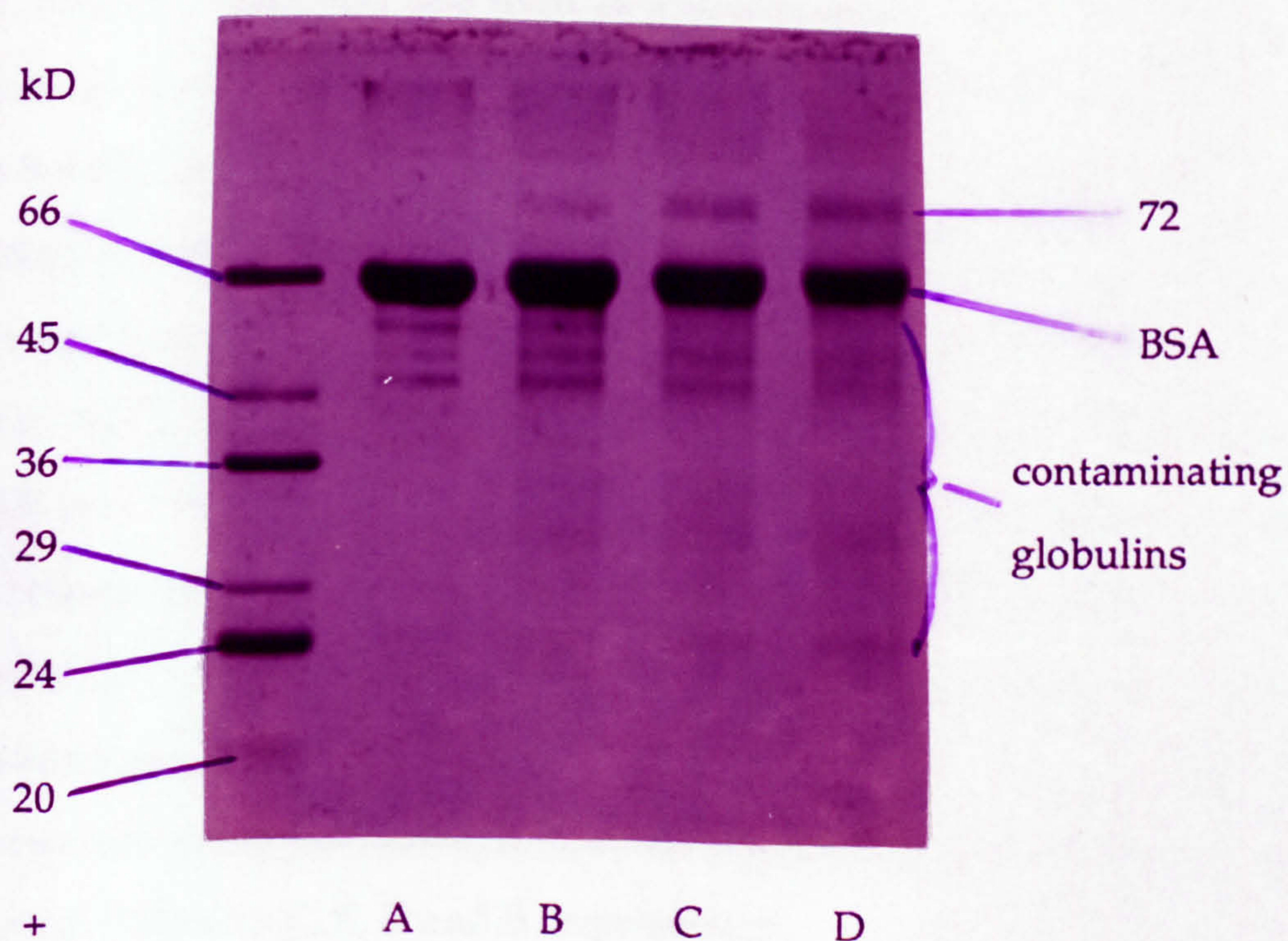
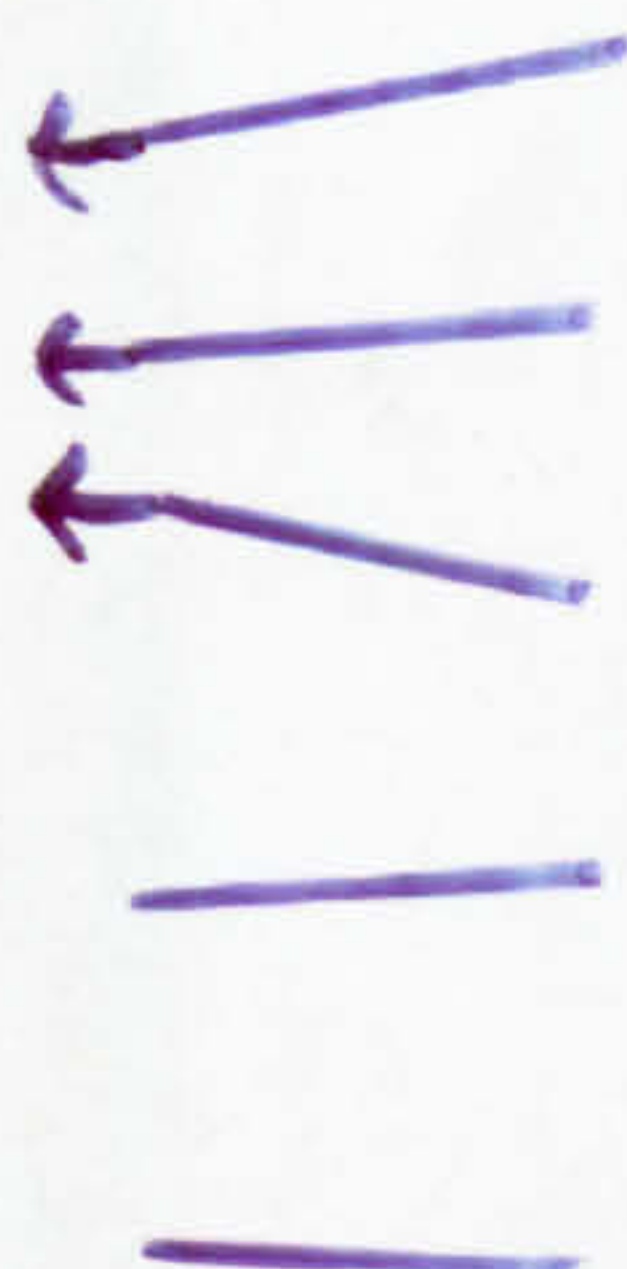


Fig 5.4.1.7. Fructation of BSA (fraction V) produces a 72kD protein band after 7 days incubation in 0.05M fructose. Lanes A, B, C and D represent 0, 7, 14 and 21 days fructation respectively. This gel was stained with Bio-Rad silver stain plus (page 65).



#### 5.4.2. Inhibition of cross-linking

The inhibition of AGE-cross-linking was studied by including aminoguanidine or the metal chelator diethylenetriaminepenta-acetic acid (DTPA) in the phosphate buffer. HEPES buffer was also used as a substitute for phosphate, because it has free radical scavenging properties and low free metal contamination. The gel in figure 5.4.2.1. and its densitometric analysis (Fig. 5.4.2.2.) illustrates the effective inhibition of cross-linking by these 3 compounds. Comparison of lanes A and B (before and after 7 days fructation respectively) reveals production of homodimers (28 and 36kD), a heterodimer (36kD) and high molecular weight aggregates (>40kD) as a result of fructation. HEPES, aminoguanidine and DTPA (lanes C, D and E respectively) all inhibit the formation of these oligomers (Fig. 5.4.2.2. depicts the heterodimer in particular). Figure 5.4.2.3. illustrates similar results for the pentosidine-linked fluorescence of the same samples (Fig. 5.4.2.1.); HEPES, DTPA and especially aminoguanidine, inhibit development of fluorescence compared to the control (columns C, E, D and B respectively).





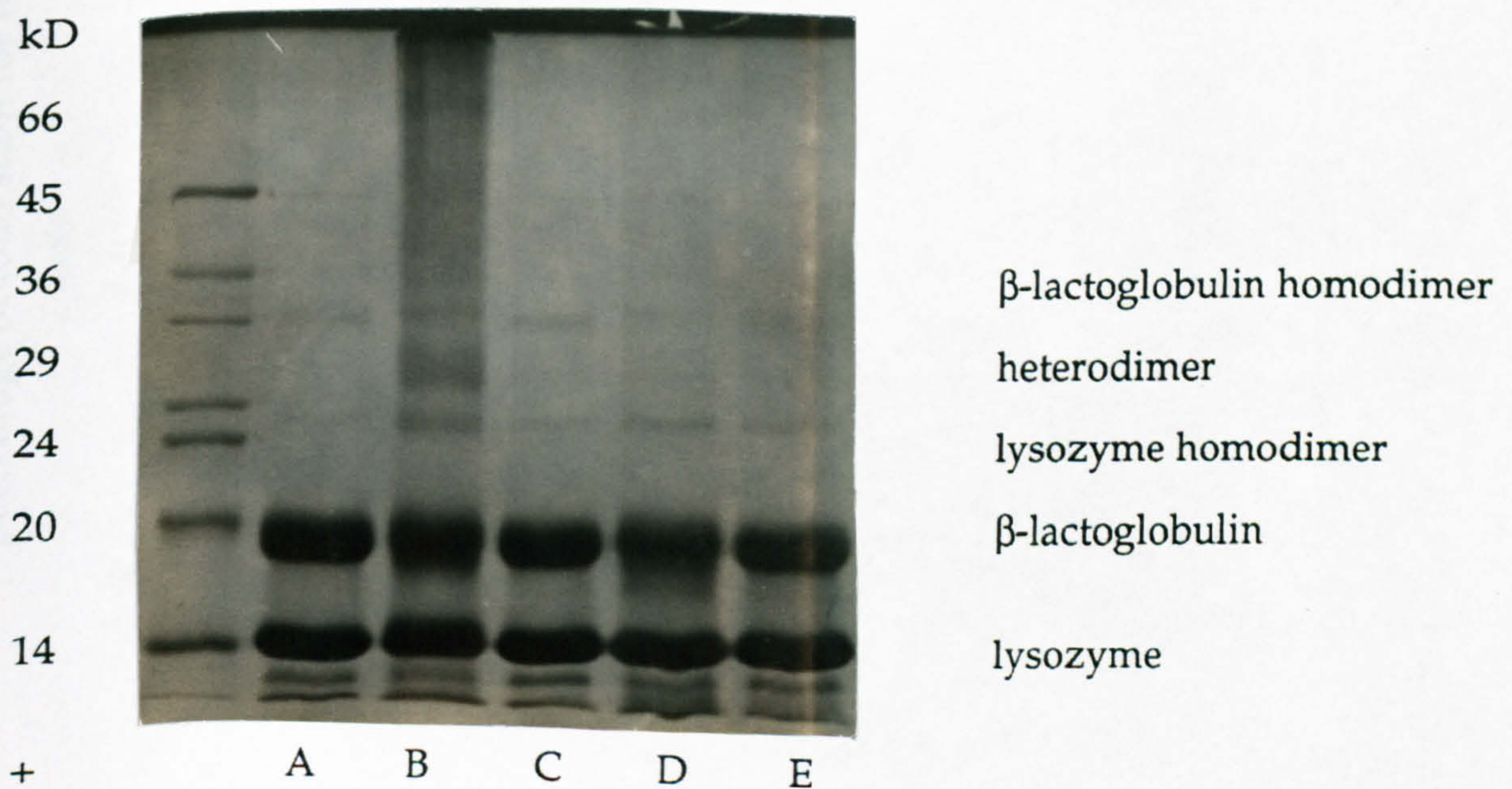


Fig 5.4.2.1. Lysozyme and  $\beta$ -lactoglobulin fructated (0.1M fructose) together with various inhibitors of AGE formation. Lane A is the two proteins before 7 days fructation, while lane B is after. Lanes C to E are also after 7 days fructation: in C the phosphate buffer has been substituted with HEPES; the samples in lanes D and E are in phosphate buffer but 50mM aminoguanidine (D) or 1mM DTPA (E) are also included.



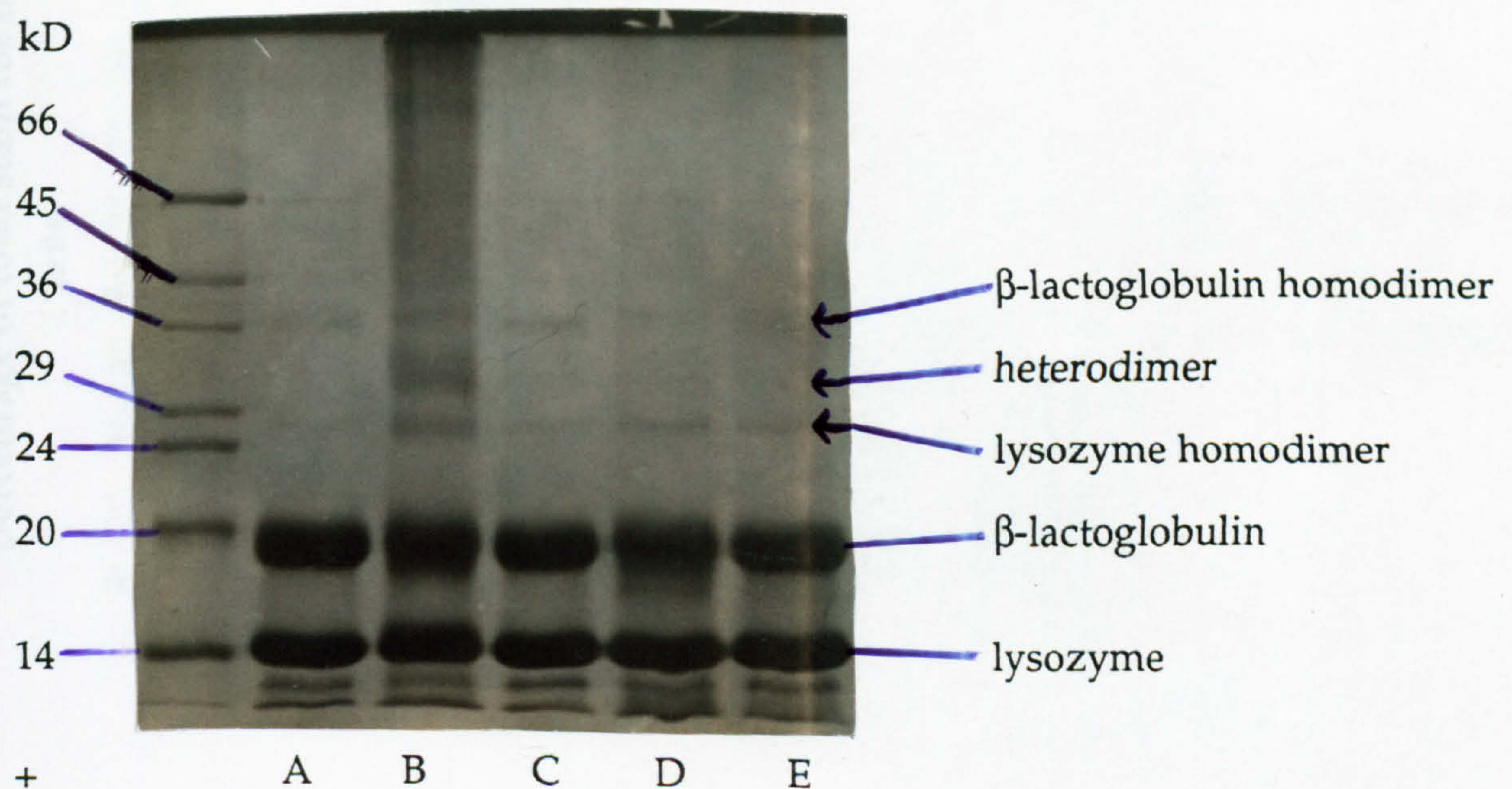


Fig 5.4.2.1. Lysozyme and  $\beta$ -lactoglobulin fructated (0.1M fructose) together with various inhibitors of AGE formation. Lane A is the two proteins before 7 days fructation, while lane B is after. Lanes C to E are also after 7 days fructation: in C the phosphate buffer has been substituted with HEPES; the samples in lanes D and E are in phosphate buffer but 50mM aminoguanidine (D) or 1mM DTPA (E) are also included.



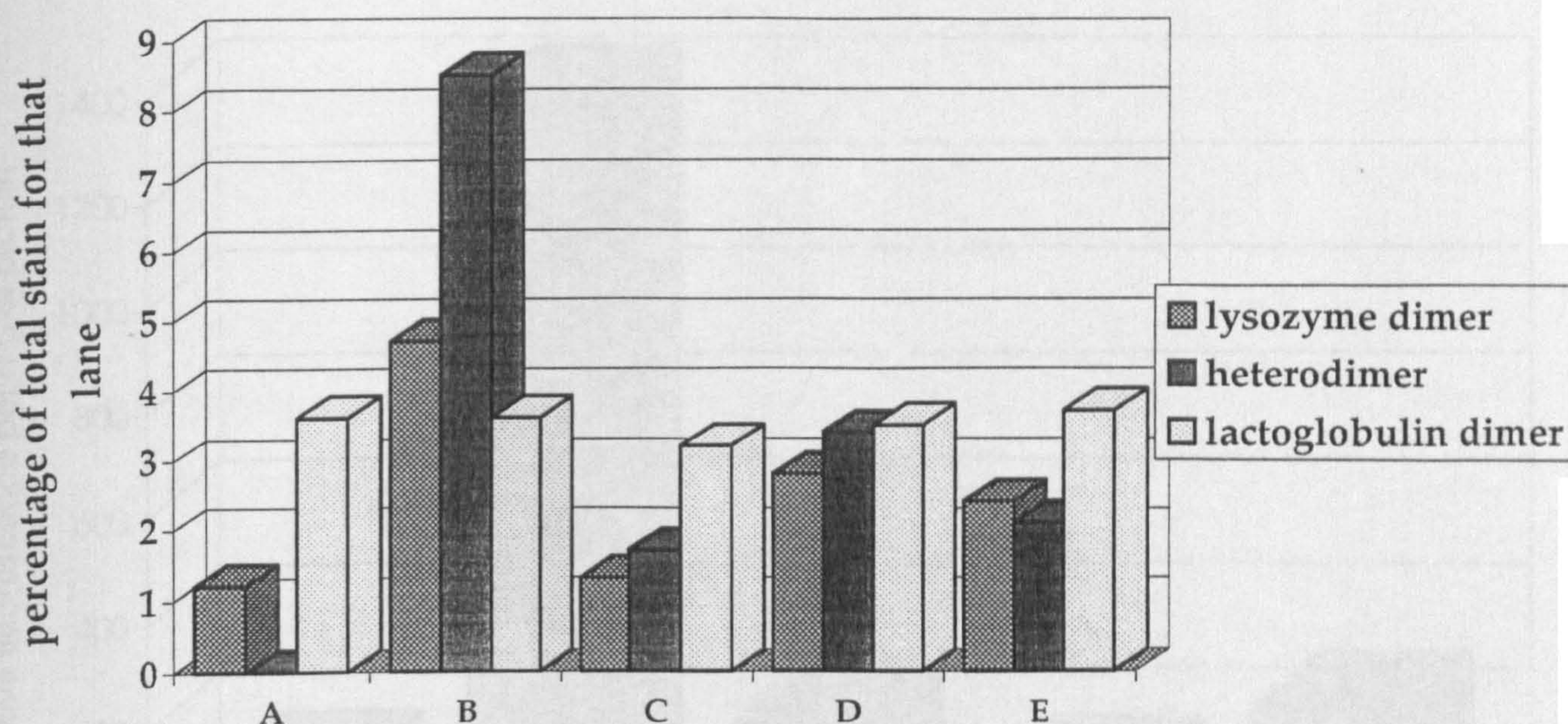


Fig. 5.4.2.2. Densitometry of the gel depicted in Figure 5.4.2.1. Inhibition of cross-linking between lysozyme and  $\beta$ -lactoglobulin after 7 days incubation with 0.1M fructose. Column A is the two proteins before 7 days fructation, while column B is after. Columns C to E are also after 7 days fructation: in C the phosphate buffer has been substituted with HEPES; the samples in columns D and E are in phosphate buffer but 50mM aminoguanidine (D) or 1mM DTPA (E) are also included.



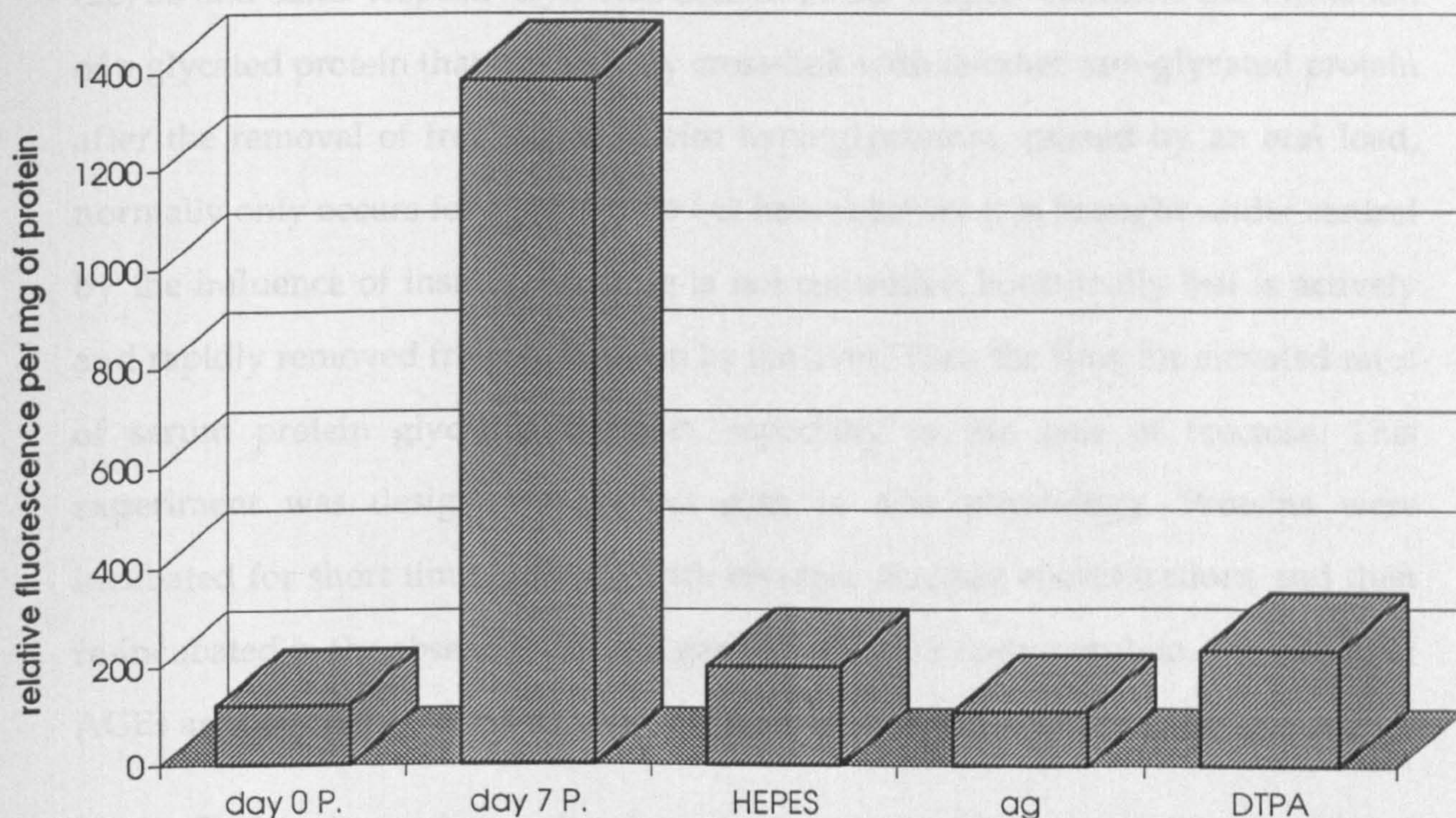


Fig. 5.4.2.3. Inhibition of pentosidine-linked fluorescence. Column A is the two proteins before 7 days fructation, while column B is after. Columns C to E are also after 7 days fructation: in C the phosphate buffer has been substituted with HEPES; the samples in columns D and E are in phosphate buffer but 50mM aminoguanidine (D) or 1mM DTPA (E) are also included.



#### 5.4.3. Investigation of cross-linking as a post-Amadori or post-Heyns reaction

The previous experiments of this chapter have shown that when lysozyme and  $\beta$ -lactoglobulin are fructated together, they form 2 homodimers and a heterodimer (28, 36 and 32kD respectively). This section of the chapter concerns the formation of a glycated protein that can actively cross-link with another non-glycated protein after the removal of free sugar. *In vivo* hyperglycaemia, caused by an oral load, normally only occurs for a short time (<2 hours) before it is brought under control by the influence of insulin. Fructose is not controlled hormonally but is actively and rapidly removed from circulation by the liver. Thus the time for elevated rates of serum protein glycation is short, especially in the case of fructose. This experiment was designed to reflect such *in vivo* physiology. Proteins were incubated for short time intervals with elevated fructose concentrations and then re-incubated in the absence of free sugar with another native protein. Formation of AGEs are detected by cross-linking of fructated protein to non-glycated protein.

Figure 5.4.3.1. shows the results of one such experiment. Lysozyme was fructated for between 0 and 8 days whereupon free fructose was removed by exhaustive dialysis against bicarbonate buffer (see page 57). The lysozyme that had been fructated to varying degrees (measured by the time of initial fructation), was then re-incubated at 37°C for 7 days with native  $\beta$ -lactoglobulin. Lane C in figure 5.4.3.1. illustrates the cross-linking between lysozyme (fructated for 2 days) and native  $\beta$ -lactoglobulin, producing a 32kD heterodimer. The heterodimer is not produced in the sugar-free control (lane A) in which lysozyme (incubated on its own for 8 days in phosphate buffer) and  $\beta$ -lactoglobulin were incubated together in bicarbonate buffer for a further 7 days. Lanes D to F show that the heterodimer is produced with greater intensity if the lysozyme is fructated for longer (4 to 8 days respectively).



Figure 5.4.3.2. shows the results of a similar experiment using native BSA. Lysozyme that had been glycated for 7 days with 0.5M fructose, was separated from the free fructose by exhaustive dialysis. The fructated lysozyme was then re-incubated in phosphate buffer with native BSA (fraction V) for between 0 and 8 days. The gel in figure 5.4.3.2. shows the typical results of this experiment with non-enzymatic modification of the BSA preparation. The gel illustrates production of a 71kD protein band, the yield of which is dependent on the time of exposure of the BSA to fructated lysozyme. A 5kD contaminant of the lysozyme preparation would have been removed by dialysis, thus the 71kD protein could not be a cross-link between BSA and such a contaminant. The 71kD protein is, however, of remarkably similar molecular weight to a 72kD band produced when BSA is fructated, as shown in figure 5.4.1.7. It is conceivable that the 71kD protein is a cross-link between lysozyme and a contaminant of the BSA preparation. Alternatively, a free cross-linking agent, such as 3-deoxyglucosone, derived from fructated lysozyme, could cross-link the contaminants to each other or BSA.

Fructated lysozyme is not exclusive in its ability to cross-link with non-glycated protein. Figure 5.4.3.3. shows the results of sugar-free re-incubation of native lysozyme with fructated  $\beta$ -lactoglobulin (0.1M fructose for 7 days in phosphate buffer). Lane A is the sample of native lysozyme and fructated  $\beta$ -lactoglobulin before re-incubation; lanes B and C are the samples after re-incubation for 4 and 8 days at 37°C. The 32kD lysozyme- $\beta$ -lactoglobulin heterodimer is formed after 4 days of re-incubation. Thus glycation of either the lysozyme or  $\beta$ -lactoglobulin will, upon sugar-free re-incubation, form the heterodimer with non-glycated protein.

All the figures presented so far, in this chapter, have been prepared from samples glycated for days at a time. I wanted to reduce the time of fructation, thus out of

the experiments presented in this section so far, I chose to incubate native  $\beta$ -lactoglobulin with fructated lysozyme. This is because this experiment produced distinct heterodimer bands at 32kD (see Fig. 5.4.3.1.). Figures 5.4.3.4. through to 5.4.3.7. are the results of a series of such experiments designed to reduce the time of fructation and yet still be able to detect AGEs. Figure 5.4.3.4. shows the results of an experiment where lysozyme was incubated with 0.5M fructose for between 0 and 90 minutes. The free fructose was removed by a rapid method (<2 hours) using size exclusion chromatography and ultrafiltration (see section 2.4.). The separated, fructated lysozyme was then re-incubated at 37°C in phosphate buffer, with native  $\beta$ -lactoglobulin, for 7 days. There is staining in the 32kD heterodimer regions of lanes B and C (Fig. 5.4.3.4.), due to cross-linking of native  $\beta$ -lactoglobulin to lysozyme fructated for 30 and 90 minutes, however no distinct bands were formed.

The gel in figure 5.4.3.4. showed very low amounts of heterodimer in comparison to the monomer, thus another experiment to investigate the minimal conditions of fructation necessary for cross-linking was devised. Figure 5.4.3.5. shows the results of that experiment. Lysozyme was fructated in phosphate buffer for up to 24 hours and exhaustively dialysed against bicarbonate buffer. During the first 12 hours of dialysis the fructose concentration, in the dialysis buffer, gradually increased from zero to 5mM (the fructose originated from the samples). Therefore all of the samples, including what had been the sugar-free control, were exposed to 5mM fructose for up to 12 hours at 4°C in bicarbonate buffer and were thus minimally fructated. Some of the sugar-free control had not been dialysed and so could still be used as the true control (lanes A), but the control sample that had been dialysed now served as a useful marker of minimal fructation (lanes B). Lanes C to L are samples where the lysozyme was fructated (0.5M fructose in phosphate buffer). for between 30 minutes and 24 hours, before free fructose was removed by exhaustive



dialysis (including the minimal fructation). All of the samples were then re-incubated in sugar-free bicarbonate buffer with native  $\beta$ -lactoglobulin. Note that the lanes are in pairs, the left lane of each pair is the sample before sugar-free re-incubation and the right is after.

The results of the gel of figure 5.4.3.5. reveal a distinct protein band at 32kD that corresponds to the lysozyme- $\beta$ -lactoglobulin heterodimer in the sample that contains extensively fructated lysozyme, i.e. was fructated for 24 hours (right lane of L). The heterodimer can, however, be clearly made out even in the sample that was minimally fructated (right lane of B). Figure 5.4.3.6. illustrates the increase in heterodimer band intensity through the range of samples, as a function of the time of the initial fructation of the lysozyme. The right lane of each pair of lanes on the gel (Fig. 5.4.3.5.) was scanned with a densitometer and the amount of heterodimer in the completely sugar-free control was subtracted from that of each sample, this ensured that only *in vitro* glycation was measured. The cross-linking observed in the sugar-free control (right lane of A) is probably between *in vivo* glycated  $\beta$ -lactoglobulin and the lysozyme.

Observation of the heterodimer in the gel and its quantification by densitometry (Figs 5.4.3.5. and 5.4.3.6.) shows that the minimally fructated sample (lane B on the gel or point bisecting the 'Y-axis' of the graph) contains around 1% of its total protein in the form of the lysozyme- $\beta$ -lactoglobulin heterodimer. The percentage of heterodimer steadily increases with the degree of fructation of the lysozyme (measured in hours of fructation). There is a change, in the slope of the graph, at around 5 hours of initial fructation, the more extensively fructated lysozyme appears to produce heterodimer more slowly than the less fructated lysozyme.

Figure 5.4.3.7. shows the net pentosidine-linked fluorescence, due to *in vitro* fructation, of the same samples used in the gel of figure 5.4.3.5.; the lysozyme only

shows a very slight increase in fluorescence even after 24 hours of fructation. The same samples, after dialysis and addition of  $\beta$ -lactoglobulin but before re-incubation, show very little difference in fluorescence. This illustrates the low rate of AGE formation during dialysis; the slight drop in fluorescence of the sample that contains lysozyme that was fructated for 24 hours, after dialysis, may be due to quenching by the added  $\beta$ -lactoglobulin or it could be due to experimental error. The fluorescence after 7 days re-incubation at 37°C in bicarbonate buffer is vastly changed, all the re-incubated samples (including 'minimally fructated') exhibit greater fluorescence than before re-incubation. The fluorescence of the samples containing lysozyme fructated for four or more hours, is proportional to the time for which the lysozyme was fructated. Between thirty minutes and four hours of fructation of lysozyme, there is no change in fluorescence; this may indicate that a certain concentration of fructated lysozyme is necessary before fluorescent AGEs will form.



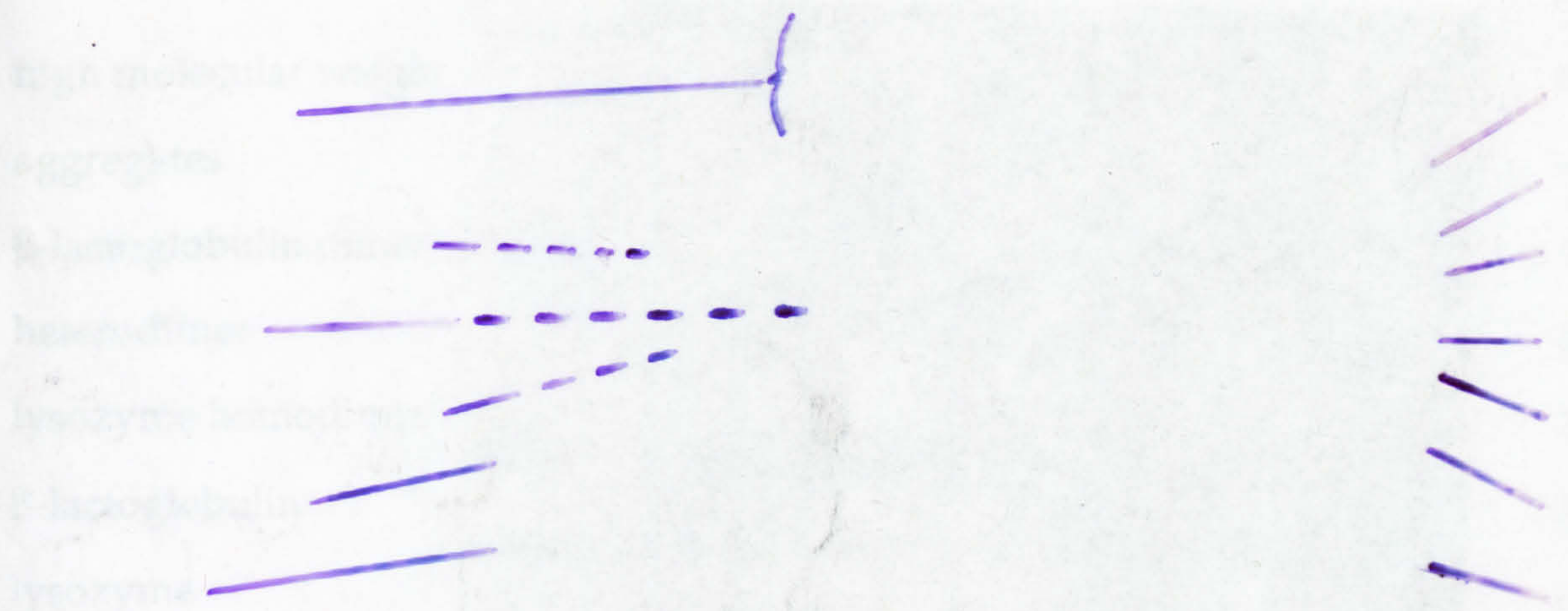


Fig. 1.3.1. Fractionation of

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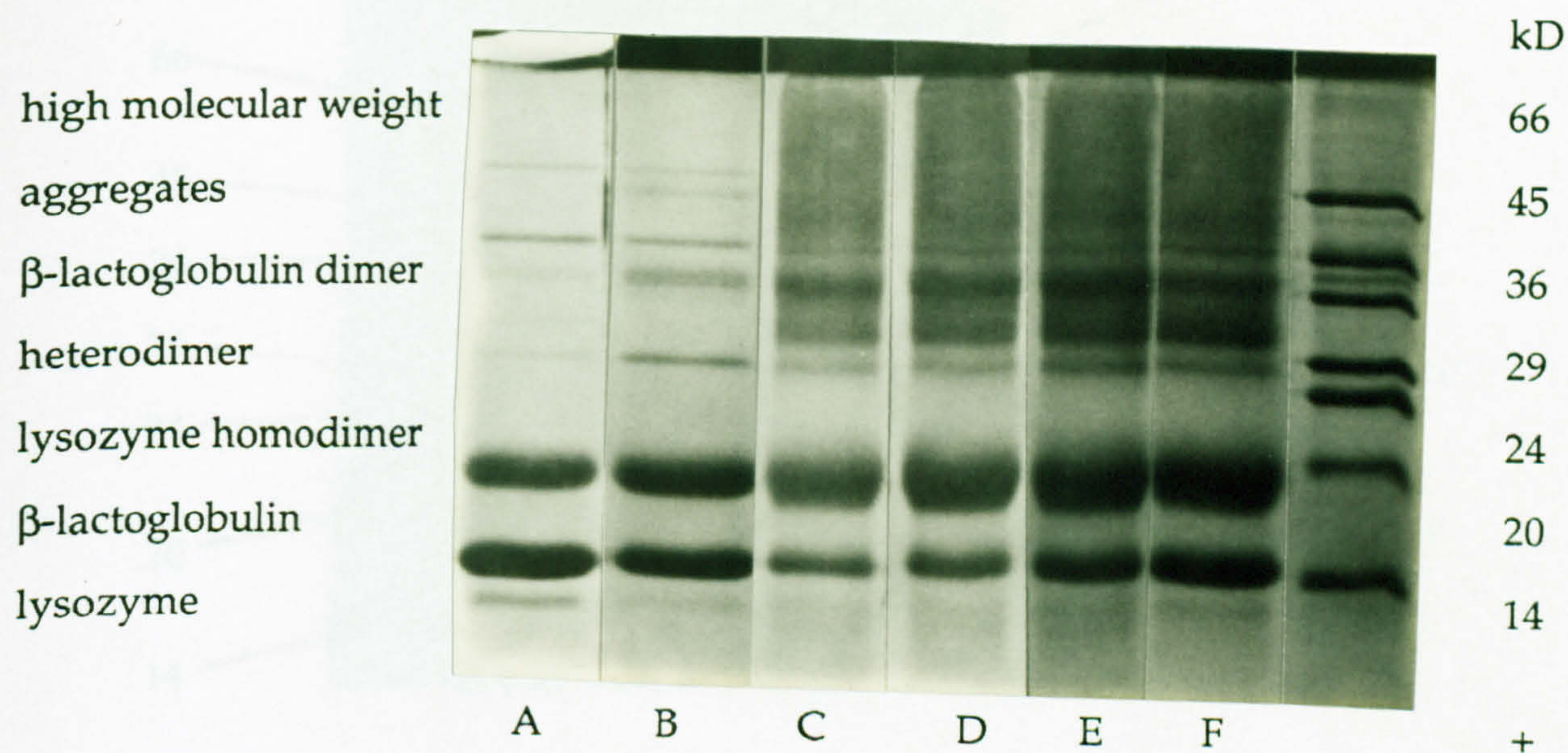


Fig 5.4.3.1. Fructated lysozyme cross-links with native  $\beta$ -lactoglobulin upon 7 days sugar-free re-incubation in bicarbonate buffer. Lane A is the sugar-free control (see text). Lanes B, C, D, E and F contain  $\beta$ -lactoglobulin after 7 days incubation with lysozyme that had been glycated for 0, 2, 4, 6 and 8 days with 0.5M fructose in phosphate buffer.



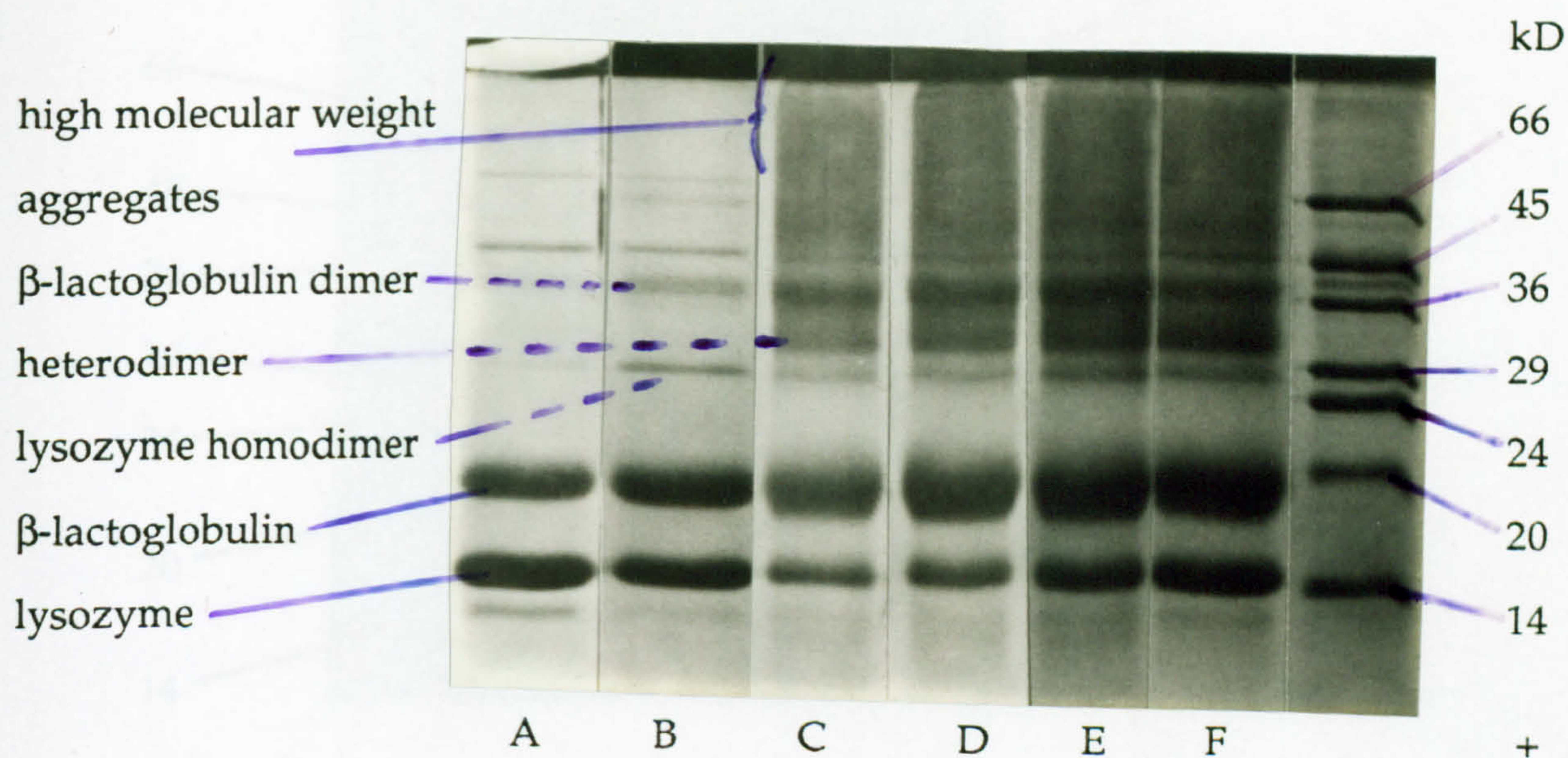


Fig 5.4.3.1. Fructated lysozyme cross-links with native  $\beta$ -lactoglobulin upon 7 days sugar-free re-incubation in bicarbonate buffer. Lane A is the sugar-free control (see text). Lanes B, C, D, E and F contain  $\beta$ -lactoglobulin after 7 days incubation with lysozyme that had been glycated for 0, 2, 4, 6 and 8 days with 0.5M fructose in phosphate buffer.



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Fig. 14.1. Purification of DNA

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buffer



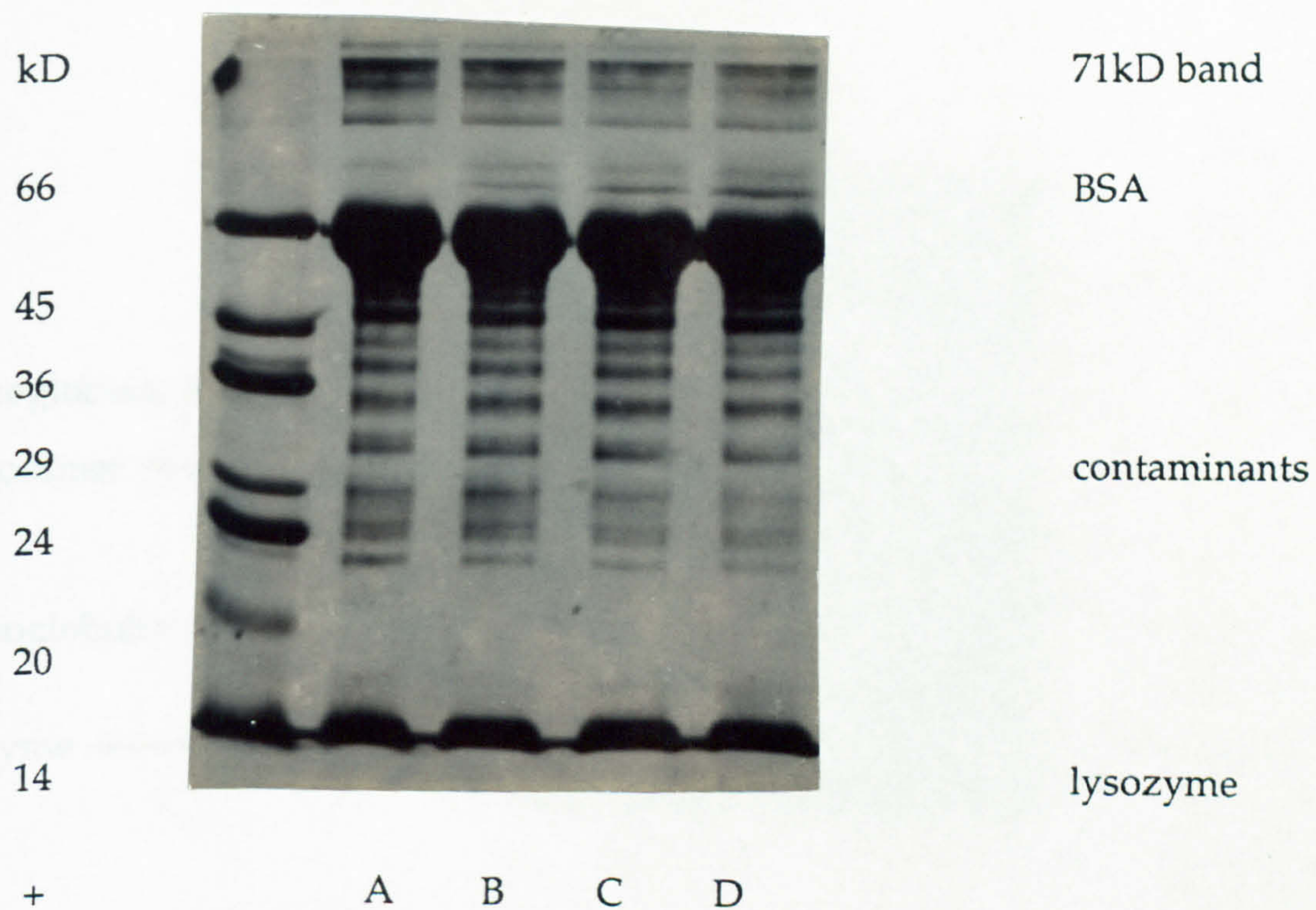


Fig 5.4.3.2. Fructated lysozyme produces a 71kD protein when incubated with native BSA for 0, 2, 6 and 8 days (lanes A to D respectively) in sugar-free phosphate buffer.



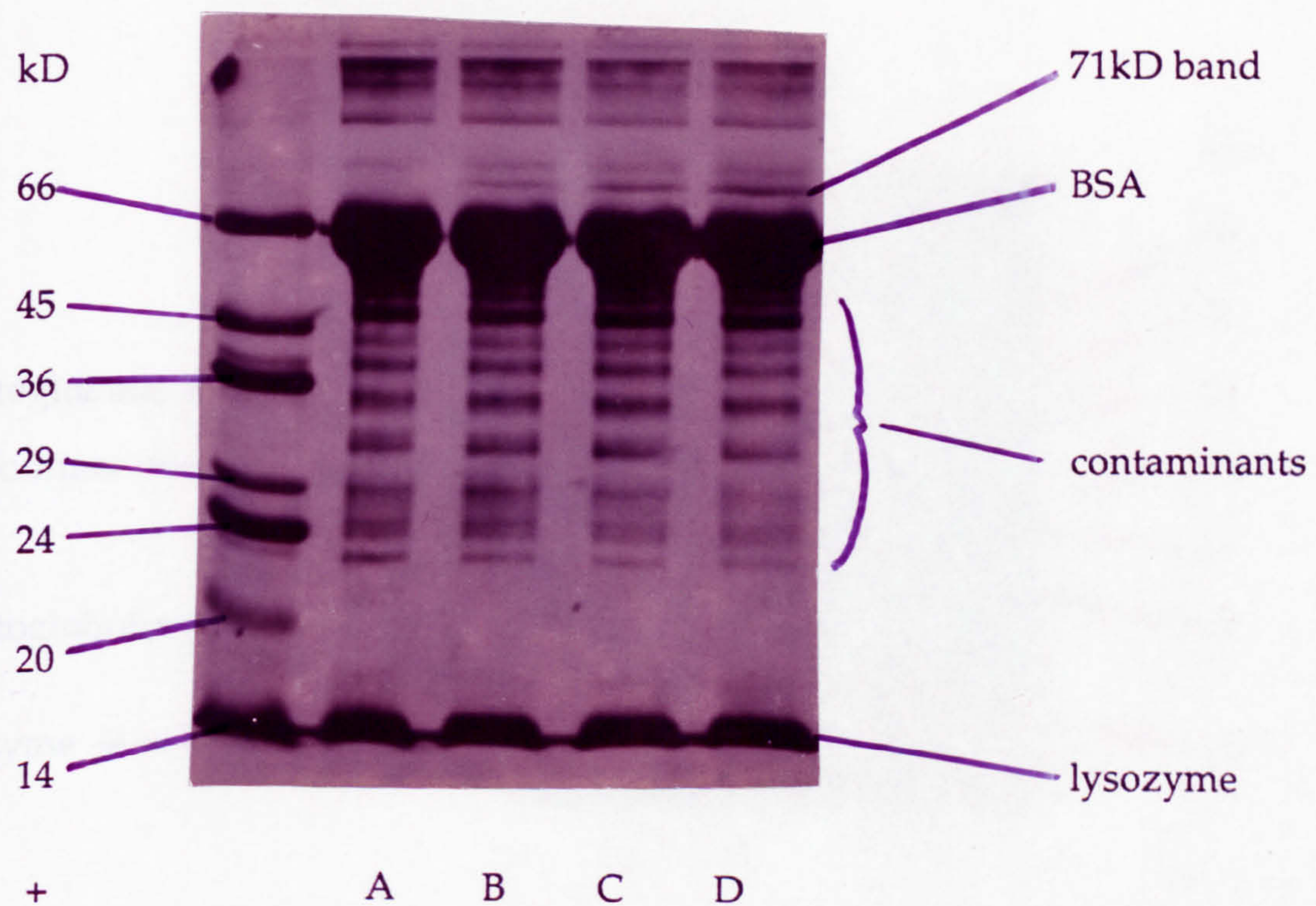


Fig 5.4.3.2. Fructated lysozyme produces a 71kD protein when incubated with native BSA for 0, 2, 6 and 8 days (lanes A to D respectively) in sugar-free phosphate buffer.



Alimentary Intestine

Colon

Stomach

Small Intestine

Fig. 13

Fig. 14

Fig. 15



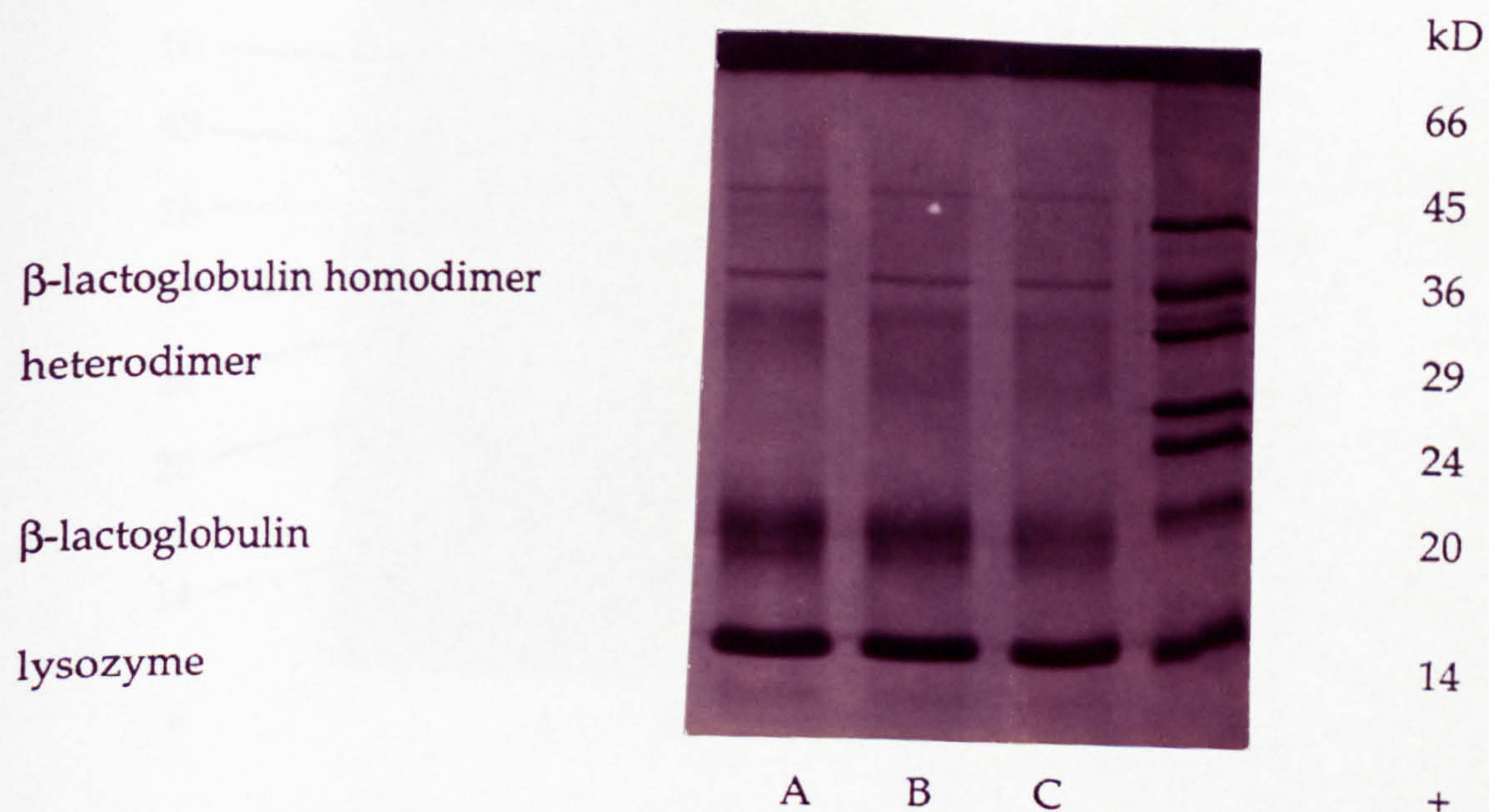


Fig 5.4.3.3. Fructated  $\beta$ -lactoglobulin (7 days, 0.1M fructose) cross-links with native lysozyme upon 0, 4 and 8 days (lanes A, B and C respectively) sugar-free re-incubation in phosphate buffer.



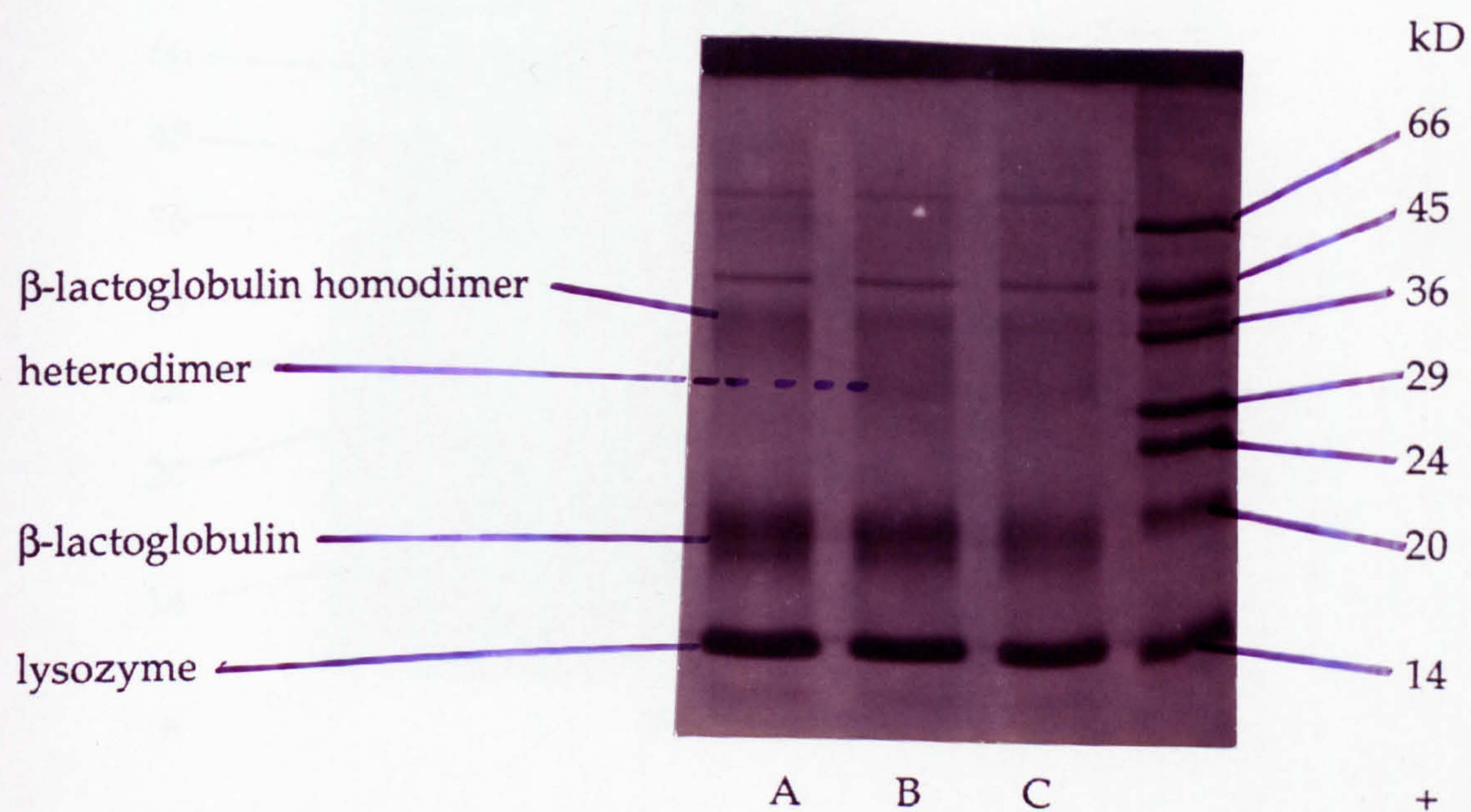


Fig 5.4.3.3. Fructated  $\beta$ -lactoglobulin (7 days, 0.1M fructose) cross-links with native lysozyme upon 0, 4 and 8 days (lanes A, B and C respectively) sugar-free re-incubation in phosphate buffer.



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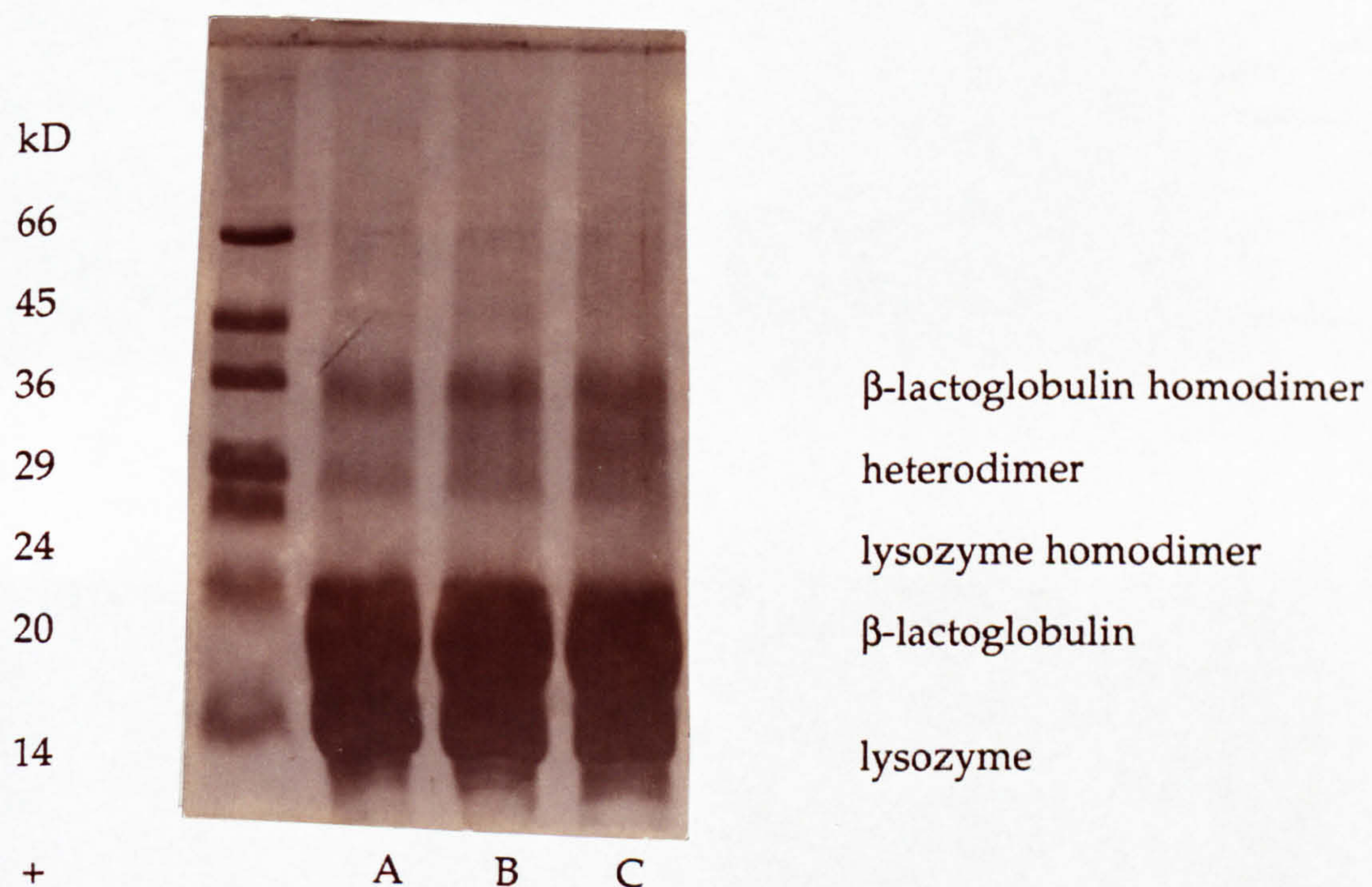


Fig 5.4.3.4. Fructated lysozyme cross-links with native  $\beta$ -lactoglobulin upon 7 days sugar-free re-incubation in phosphate buffer. Lanes A, B and C represent lysozyme that was glycated with 0.5M fructose in phosphate buffer for 0, 30 and 90 minutes respectively, prior to removal of free sugar and re-incubation with  $\beta$ -lactoglobulin.



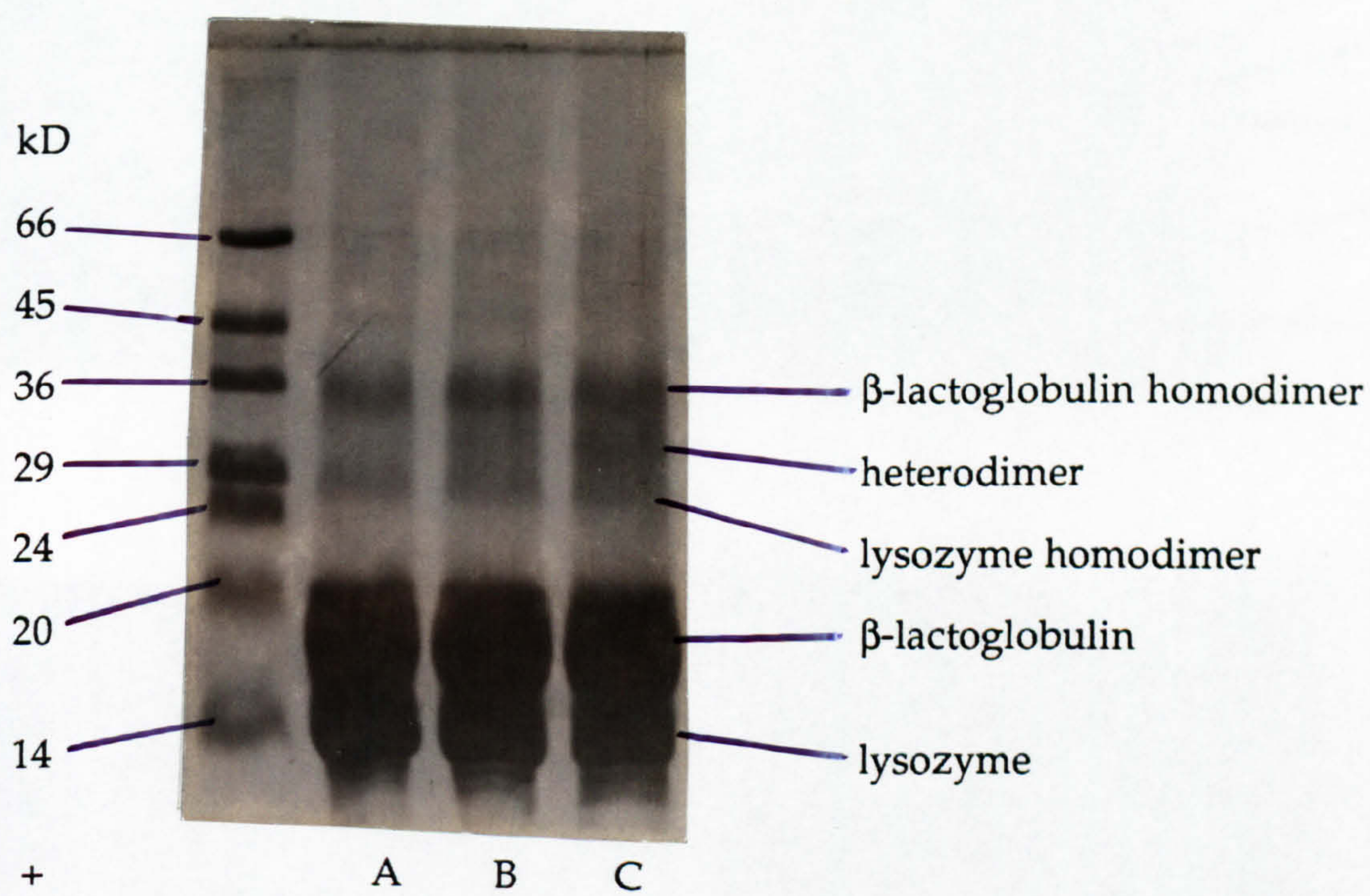


Fig 5.4.3.4. Fructated lysozyme cross-links with native  $\beta$ -lactoglobulin upon 7 days sugar-free re-incubation in phosphate buffer. Lanes A, B and C represent lysozyme that was glycated with 0.5M fructose in phosphate buffer for 0, 30 and 90 minutes respectively, prior to removal of free sugar and re-incubation with  $\beta$ -lactoglobulin.







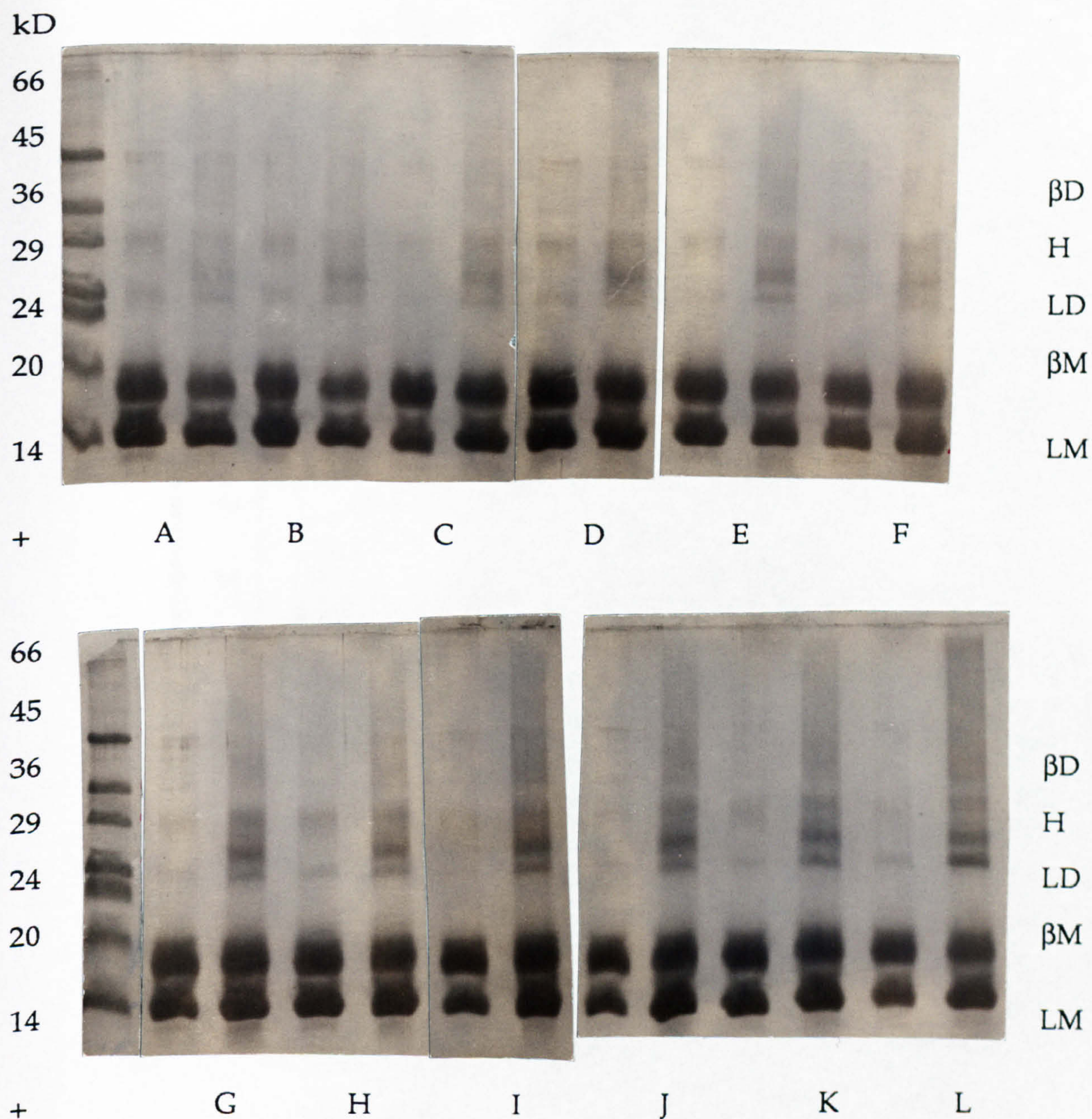


Fig 5.4.3.5. Lysozyme glycated with 0.5M fructose for 0, 0.5, 1.5, 2.5, 3, 4, 5, 7, 8, 12 and 24 hours, upon 7 days sugar-free re-incubation in bicarbonate buffer cross-links with native  $\beta$ -lactoglobulin (pairs of lanes B to L respectively). Lanes A are the completely sugar-free control. The left lane of each pair is the sample before sugar-free re-incubation and the right is after. The abbreviations represent;  $\beta$ D =  $\beta$ -lactoglobulin homodimer, H = heterodimer, LD = lysozyme homodimer,  $\beta$ M =  $\beta$ -lactoglobulin monomer and LM = lysozyme monomer.



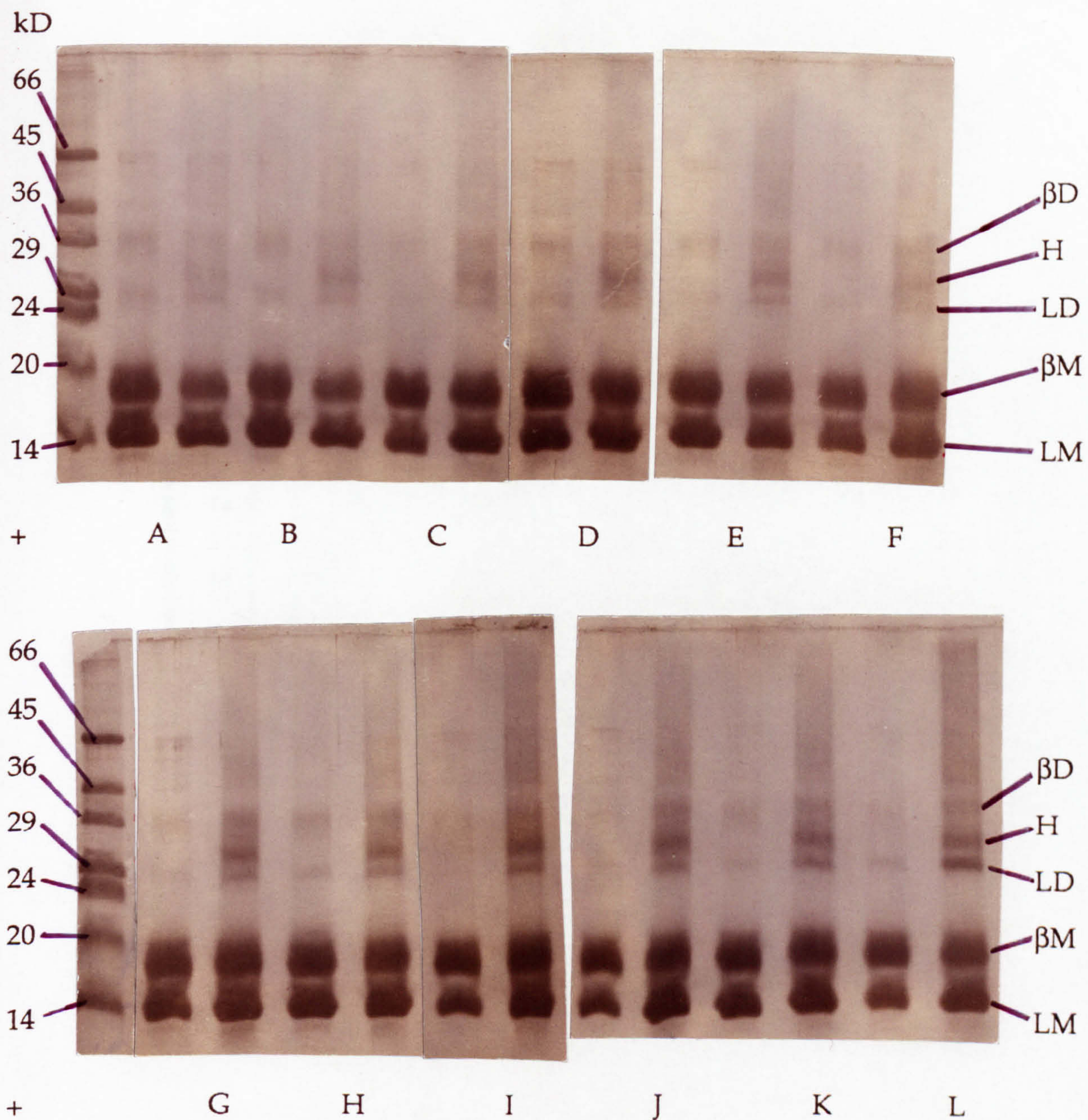


Fig 5.4.3.5. Lysozyme glycated with 0.5M fructose for 0, 0.5, 1.5, 2.5, 3, 4, 5, 7, 8, 12 and 24 hours, upon 7 days sugar-free re-incubation in bicarbonate buffer cross-links with native  $\beta$ -lactoglobulin (pairs of lanes B to L respectively). Lanes A are the completely sugar-free control. The left lane of each pair is the sample before sugar-free re-incubation and the right is after. The abbreviations represent;  $\beta$ D =  $\beta$ -lactoglobulin homodimer, H = heterodimer, LD = lysozyme homodimer,  $\beta$ M =  $\beta$ -lactoglobulin monomer and LM = lysozyme monomer.



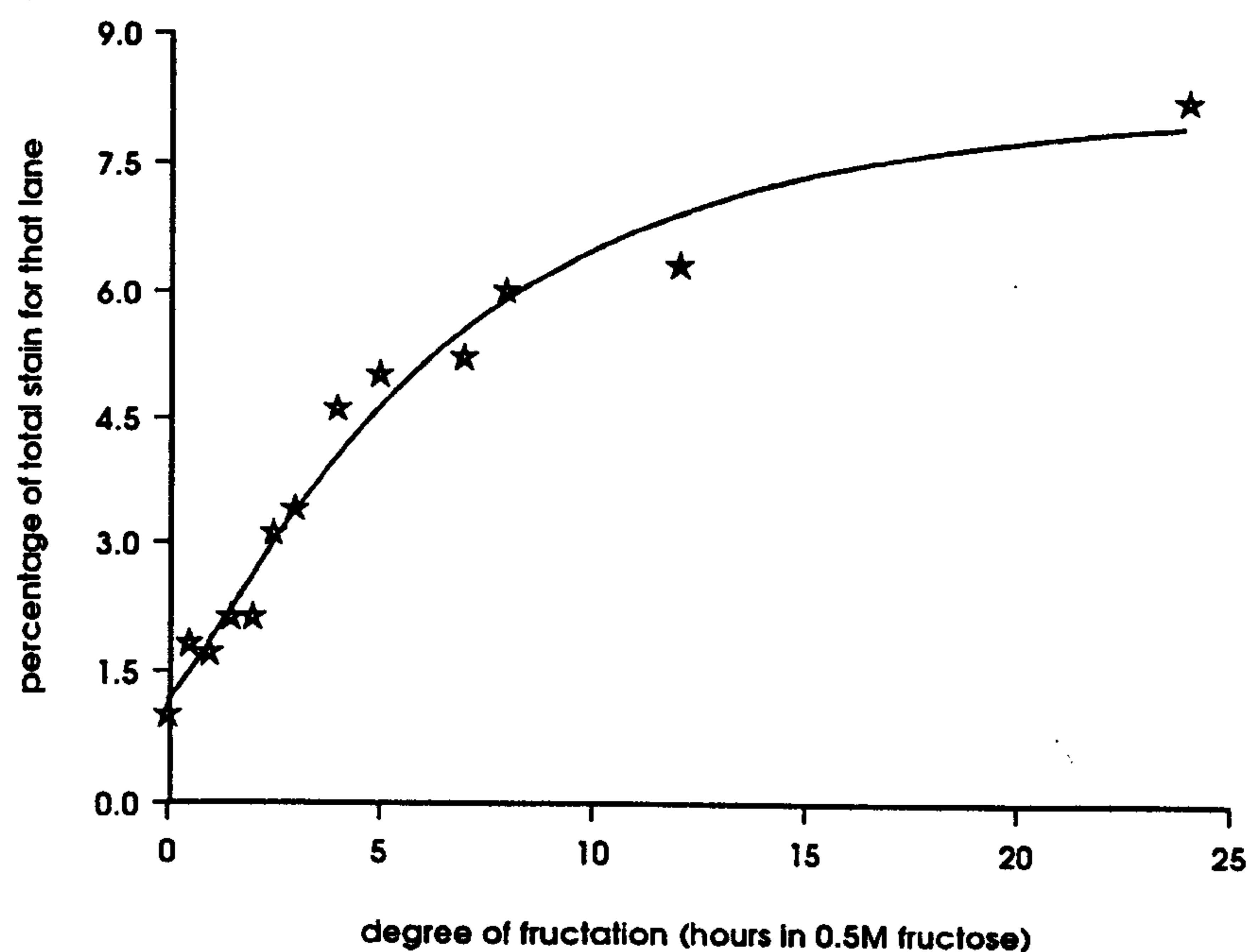


Fig. 5.4.3.6. Densitometry of the gels of Fig. 5.4.3.5.; formation of lysozyme- $\beta$ -lactoglobulin heterodimer upon 7 days re-incubation of fructated lysozyme with native  $\beta$ -lactoglobulin. The x-axis represents the time that the lysozyme was initially fructated and thus the degree of fructation. The percentage staining of the completely sugar-free control has been subtracted from that of the fructated samples, so that only *in vitro* fructation is measured.



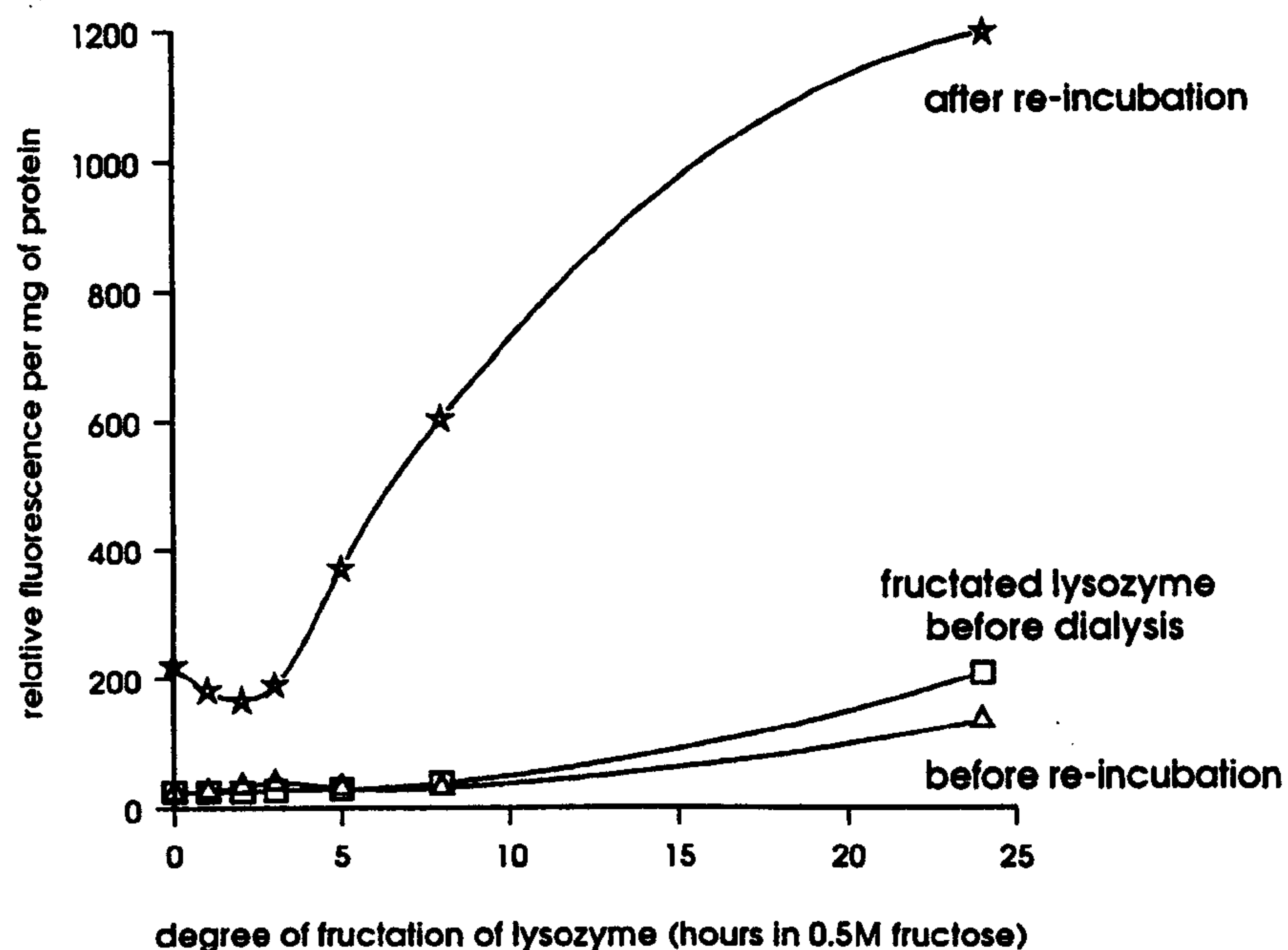


Fig. 5.4.3.7. The pentosidine-linked fluorescence of the samples used in the gel of figure 5.4.3.5. The net fluorescence of lysozyme, due to *in vitro* fructation, before dialysis is represented by the squares. The triangles represent the net fluorescence, due to *in vitro* glycation, of the dialysed fructated lysozyme after addition of  $\beta$ -lactoglobulin (left lanes in each pair of Fig 5.4.3.5.); while the stars represent the net fluorescence of the same samples after 7 days sugar-free re-incubation (right lanes in each pair of Fig. 5.4.3.5.).

## 5.5. Discussion

The study of Maillard cross-linking is important to increase our understanding of the biochemical basis of the pathogenesis of diabetic secondary complications *in vivo*. If diabetic secondary complications are representative of accelerated ageing, greater knowledge of the Maillard reaction would also increase our comprehension of the normal ageing process. It is likely that conjugation of two different proteins *in vivo* would alter their activity, function, receptor-mediated uptake and turnover. Thus Maillard cross-linking *in vivo* has a great potential for alteration of the function of tissues and production of degenerative diseases.

### 5.5.1. Fructation-induced cross-linking

The faster rate of cross-linking of lysozyme, RNase and collagen upon fructation compared to glucation (at equal sugar concentrations) has been reported previously [198, 199, 200 and 133]. The results contained in this thesis agree with those results and add  $\beta$ -lactoglobulin to the list of proteins studied. Fructation of lysozyme or  $\beta$ -lactoglobulin produces intermolecular AGE-cross-links, resulting in oligomers (Figs. 5.4.1.1. and 5.4.1.4.). Also, if a solution of lysozyme and  $\beta$ -lactoglobulin is fructated, they will cross-link to form a 32kD heterodimer, figure 5.4.1.6. The lysozyme preparation is contaminated by two proteins that are clearly stained in figure 5.4.1.1.; ovalbumin at 45kD and an unknown contaminant at about 73kD. The  $\beta$ -lactoglobulin preparation is contaminated by its own dimer (36kD) which is likely to have been produced from *in vivo* glycation by lactose since the protein is purified from bovine milk.

BSA is not an ideal protein to use when studying cross-linking by SDS-PAGE because of its high molecular weight, i.e. dimers and trimers of BSA have



molecular weights in the region of the gel that has a non-linear gradient. Figure 5.4.1.7. is a gel of fructated BSA and shows the production of a 72kD protein band as a function of the time of fructation. The nature of the 72kD band cannot be determined using SDS-PAGE and I would have liked to have performed western blotting on it, but there was not enough time available. It is possible that the 72kD protein is formed by the cross-linking of two contaminants of the BSA preparation rather than BSA plus a 5kD oligomer. Glycation of the globulins that contaminate BSA is indicated by the spreading and fading of their stained bands as a function of the time of fructation, for example  $\beta$ -lactoglobulin monomer also fades and spreads in figure 5.4.1.4. The spreading and fading is due to impaired binding of SDS to glycated protein, which causes it to migrate in the gel behind the native form. Alternatively, the 72kD band could be BSA monomer with a number of fructose residues attached. Lapolla *et al* (1994) reported that BSA increased in molecular weight with addition of glucose residues to form a stable molecule of 70kD [115]. If however the 72kD protein were BSA with a number of fructose residues attached, I would have expected to see bands between 67 and 72kD of other BSA-fructose adducts.

#### 5.5.2. Inhibition of cross-linking

Figures 5.4.2.1. to 5.4.2.3. illustrate inhibition of AGE formation by the use of anti-oxidative conditions (HEPES and DTPA) and by sequestration of reactive groups (aminoguanidine). Substitution of phosphate buffer with HEPES was the most effective method to prevent heterodimer formation, while inclusion of DTPA or aminoguanidine in phosphate buffer does result in some cross-linking, albeit at a lower rate than without the inhibitors. It is possible that DTPA does not fully prevent the free metal of phosphate buffer from taking part in MCO [68]. This may account for the higher percentage of lysozyme dimer and heterodimer produced

upon fructation with DTPA than in HEPES buffer. The low level of AGE formation in HEPES buffer indicates that there are routes to AGE formation that are independent of MCO.

Prabhakaram and Ortwerth (1994) wrote an interesting article concerning cross-linking of glycated lysozyme to [14C]-lysine and other amino acids in the presence of 1mM DTPA [206]. Glycation using fructose, galactose and glucose gave rise to virtually no cross-linking; while 3-DOG, ascorbate, and its degradation products did produce cross-links. The fact that 3-DOG produced cross-links but fructose did not, indicates that the hypothesis of 3-DOG formation from fructose is not a reaction with a high rate under conditions similar to those *in vivo*. Alternatively, it indicates that such a conversion is free metal dependent, see section 1.7. for a review of 3-DOG production from fructose.

The inhibition of AGE formation by aminoguanidine is interesting because the results of this chapter indicate that it completely inhibits fluorophore formation (Fig. 5.4.2.3.) but not cross-linking (Fig. 5.4.2.2.); thus cross-linking could be an earlier or more sensitive indicator of AGE formation than fluorescence. Alternatively the mechanism of production of some cross-links is different to that of fluorophore formation and aminoguanidine does not block them all. Aminoguanidine is a non-specific hydrazine and will react with any available carbonyl group that occurs in the Maillard reaction. The principle site of aminoguanidine action in the Maillard reaction is, however, contentious and several sites have been proposed. See chapter 4. for more information; briefly, aminoguanidine can potentially react with free sugar, Amadori or Heyns product and post-Amadori or post-Heyns intermediates to AGE formation.

Aminoguanidine can react with the acyclic aldehyde or ketone forms of free sugars inhibiting glycation. Blackledge (1993) reported the production of



chromophores from such a reaction [166]. Lane D of figure 5.4.2.1., however, illustrates a spreading and fading of the  $\beta$ -lactoglobulin monomer in the presence of aminoguanidine, as a function of the time of fructation,. while AGE formation in the same samples is blocked; this indicates that aminoguanidine has a low reactivity with free sugar in comparison to a product of glycation. This effect of inhibiting AGE formation, but not glycation, by aminoguanidine, supports the theory that it reacts with post-Amadori or post-Heyns compounds preventing their further reaction, which would otherwise produce AGEs. This theory was proposed by Fu *et al* (1992) and supported by evidence presented in chapter 4. [134].

The conclusions drawn from the inhibitor experiments of this chapter are used to modify figure 1.1. and produce figure 5.5.2.1.

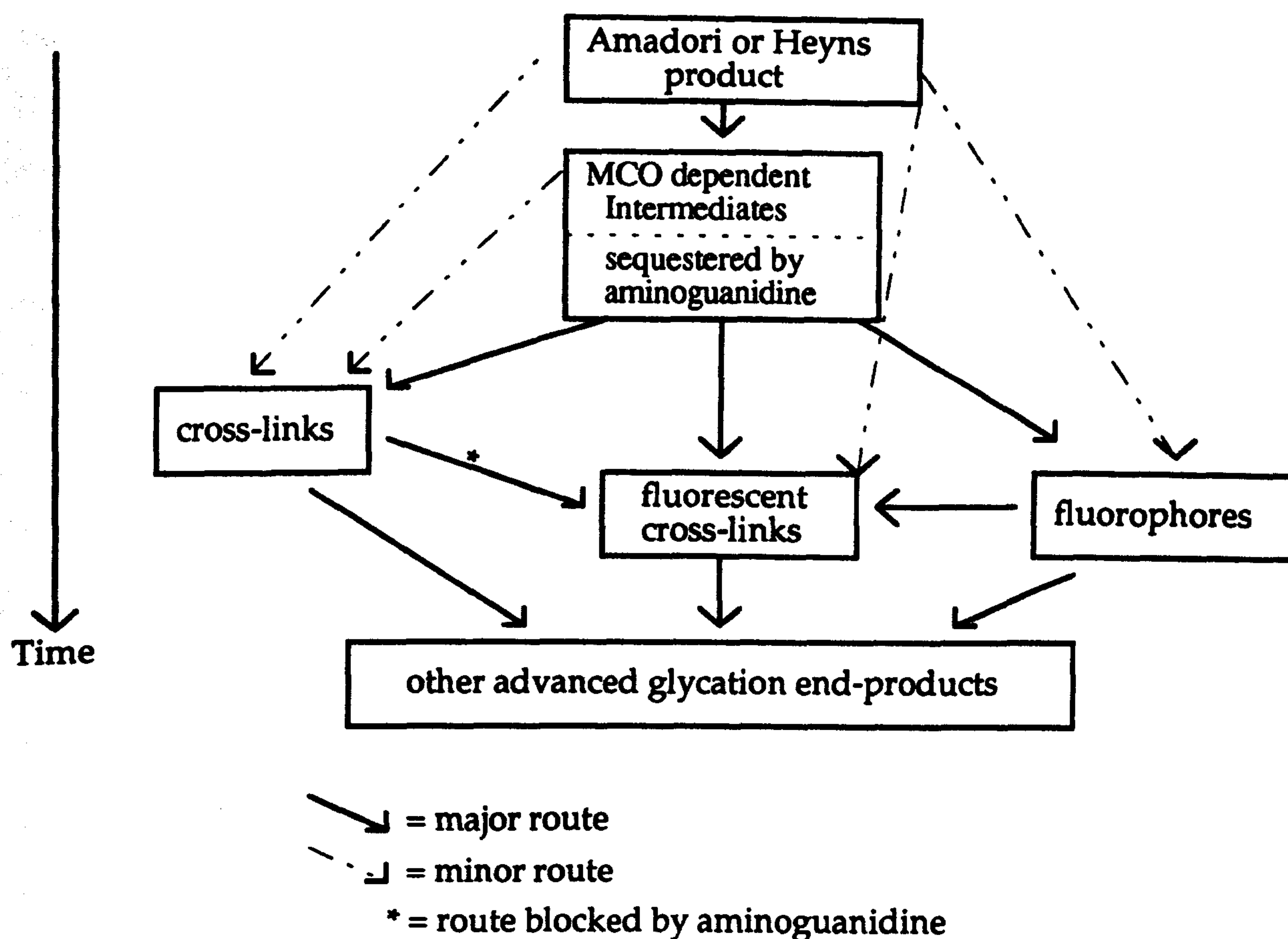


Fig. 5.5.2.1. Schematic diagram of the possible routes for post-Amadori or post-Heyns reactions and their inhibition by DTPA and aminoguanidine.

### 5.5.3. Investigation of cross-linking as a post-Amadori reaction

The third part of the results section of this chapter shows how glycated protein can form cross-linked and fluorescent AGEs with non-glycated proteins in the absence of free sugar. *In vivo* elevated monosaccharide concentrations, in circulation, are caused by ingestion of a carbohydrate rich meal. Glucose is removed from circulation under the influence of insulin throughout the body and normal concentrations of ingested fructose are removed by the liver before the blood in the hepatic portal vein enters general circulation. Thus proteins throughout the



body can be exposed to hyperglycaemia, but only those in the blood of the hepatic portal vein and surrounding tissues are exposed to short periods of elevated concentrations of dietary fructose. It is conceivable that the rate of fructation is swift enough such that dietary fructose could produce stable glycated protein that would contribute to diabetic secondary complications and ageing in the general population.

Figure 5.4.3.1. shows that fructated lysozyme will cross-link with native  $\beta$ -lactoglobulin upon 7 days sugar-free re-incubation and form a very sharp lysozyme- $\beta$ -lactoglobulin heterodimer band (32kD) that increases in intensity if the lysozyme is fructated for longer periods. Figure 5.4.3.3. illustrates that fructated  $\beta$ -lactoglobulin will cross-link to native lysozyme and form the 32kD heterodimer after 4 days of sugar-free re-incubation in phosphate buffer. Thus glycation of either lysozyme or  $\beta$ -lactoglobulin produces a form of protein that will actively cross-link with the other native protein upon sugar-free re-incubation.

Figure 5.4.3.2. shows the production of a 71kD band, upon re-incubation of fructated lysozyme with native BSA, the intensity of which increases with the time of exposure of the BSA to the fructated lysozyme. The molecular weight of the 71kD band is too low for a lysozyme-BSA heterodimer ( $67 + 14 = 81\text{kD}$ ), but the band is, however, of remarkably similar molecular weight to a 72kD band produced when BSA is fructated on its own (figure 5.4.1.7.). Contaminants of the lysozyme preparation of less than 10kD would have been removed by the ultrafiltration step (removal of unreacted sugar), therefore the 71kD band could be fructated lysozyme cross-linked to a contaminant of the BSA preparation, or a post-Heyns reactive intermediate that is derived from, but free of, the fructated lysozyme. 3-DOG (page 49 *et sequa*) has been proposed to be derived from Amadori product and act as a free agent cross-link protein, thus it could be 3-DOG

that cross-links two contaminants of the BSA preparation. Alternatively compounds such as 3-DOG, or even fructose released from the fructated lysozyme, could react with the BSA, increasing its molecular weight. BSA was reported to form a stable structure at 70kD upon glucation, the structure was proposed to be BSA with around 16 glucose residues attached [115].

Further investigation of post-Amadori or post-Heyns reactions using sugar-free re-incubation revealed that as little as 30 minutes fructation of lysozyme, followed by a rapid removal of free sugar and 7 days re-incubation with  $\beta$ -lactoglobulin, produces a 32kD heterodimer (Fig. 5.4.3.4.). The heterodimer bands produced in this experiment were, however, of poor contrast. Thus the experiment was modified using dialysis into bicarbonate buffer, rather than size exclusion chromatography and ultrafiltration, to remove free sugar; the results are shown in figures 5.4.3.5. to 5.4.3.7.

Dialysis removes molecules smaller than the molecular weight cut-off of the dialysis membrane at a rate that is dependent on the concentration gradient. In the system used for experiments presented in this thesis, the volume of dialysis buffer was continually pumped across the membrane and samples, in a closed loop, such that an equilibrium between samples and dialysis buffer was reached. The final concentration of the small molecules (less than 6-8kD) in each change of buffer, and thus in the samples, was dependent on the volume of the samples, the dialysis buffer and the initial sugar concentrations. For the samples in figure 5.4.3.5. the dilution of small molecules was 100 fold for each change of buffer, thus in the first 12 hours of dialysis, before the first change of buffer, the concentration of fructose in the dialysis buffer rose to 5mM. This meant that the sample that had up to that point been the sugar-free control, was exposed to an increasing concentration of fructose (up to 5mM) for up to 12 hours at 4°C in bicarbonate buffer. Therefore,



upon completion of dialysis this sample had been 'minimally fructated' (12 hours, 5mM fructose, 4°C in bicarbonate buffer). So an aliquot of the same sample taken before dialysis, was used as the true sugar-free control for the rest of the experiment. Thus figure 5.4.3.5. illustrates the cross-linking of fructated lysozyme to native  $\beta$ -lactoglobulin to form a 32kD heterodimer upon sugar-free re-incubation in bicarbonate buffer. The sample that contained lysozyme fructated for 24 hours (right lane of L) shows a very clear heterodimer at 32kD, in a similar manner to figure 5.4.3.1., it can however be discerned in all of the samples, including the completely sugar-free control and the sample that was minimally fructated (right lanes of A and B respectively).

The densitometry of the gel in figure 5.4.3.5. (Fig. 5.4.3.6.), shows that the intensity of the heterodimer is proportional to the time that the lysozyme was initially fructated and therefore the amount of fructated lysozyme in the sample. The cross-linking observed in the true sugar-free control is probably due to *in vivo* glycation of the  $\beta$ -lactoglobulin, which was purified from pasteurised skimmed milk; cows' milk contains the reducing disaccharide lactose at a concentration of about 0.13M [207]. The time lapsed between milking to the final lyophilisation of the purified  $\beta$ -lactoglobulin was not available from Sigma, thus the degree of *in vivo* glycation will vary between batches of protein, thus this variable was removed from the experimental results presented. The densitometry and fluorescence are the net results due to *in vitro* fructation, i.e. the amount of heterodimer produced from *in vivo* glycation of  $\beta$ -lactoglobulin (sugar-free control) has been subtracted.

The increase in heterodimer formation in figures 5.4.3.5. and 5.4.3.6. is biphasic, there is an initial fast rate of production (up to 5 hours) which tapers off into a slower rate for the samples that contain more extensively fructated lysozyme. The change in rate of formation could be due to limitation of substrates; such as

available amino groups on the lysozyme during the initial fructation, or cross-linking sites on the  $\beta$ -lactoglobulin. It is also conceivable that the heterodimer is a substrate for the production of a higher molecular weight AGE, but unless this AGE was only formed when a critical amount of its substrates were present, this would not account for the change in the rate of its formation. If the heterodimer was becoming part of a higher molecular weight protein it would be lost in a similar proportion in all the samples.

The fluorescence of the samples used in the gel of figure 5.4.3.5. is illustrated in figure 5.4.3.7. The two lowest curves should, in theory, be almost the same, with perhaps some extra AGE formation in the samples that have been dialysed. The lower fluorescence of the samples after dialysis, compared to before, is probably due to quenching by the added  $\beta$ -lactoglobulin. The upper curve is the fluorescence of  $\beta$ -lactoglobulin and fructated lysozyme after 7 days sugar-free re-incubation. The fluorescence after re-incubation is dependent on the time of initial fructation. There is very little difference in the fluorescence of the samples containing lysozyme fructated for less than 3 hours, whereas samples containing lysozyme fructated for longer than 3 hours exhibit a fluorescence that is dependent on the time for which the lysozyme was fructated. This implies that a certain concentration of fructated lysozyme is necessary before fluorophores will form.

Comparison of the cross-linking and fluorescence of the same re-incubated samples (Figs. 5.4.3.6. and 5.4.3.7.) illustrates that at first cross-linking forms faster than the development of fluorescence. When the lysozyme has been fructated more extensively (four or more hours), however, the rates reverse. Highly fructated lysozyme produces more fluorescence than cross-links with  $\beta$ -lactoglobulin. The threshold of the change from formation of cross-linked AGE to fluorescent AGE is when the lysozyme has been fructated for 4 to 5 hours. The



results suggest common post-Heyns compounds, that are intermediates in the production of cross-linked and fluorescent AGEs. The concentration of these, and probably other post-Heyns (or post-Amadori if the reactions are analogous), is critical to the particular AGEs formed; below a certain concentration cross-links are formed, above it fluorophores are formed. This conclusion is illustrated in the simple schematic diagram in figure 5.5.3.1.

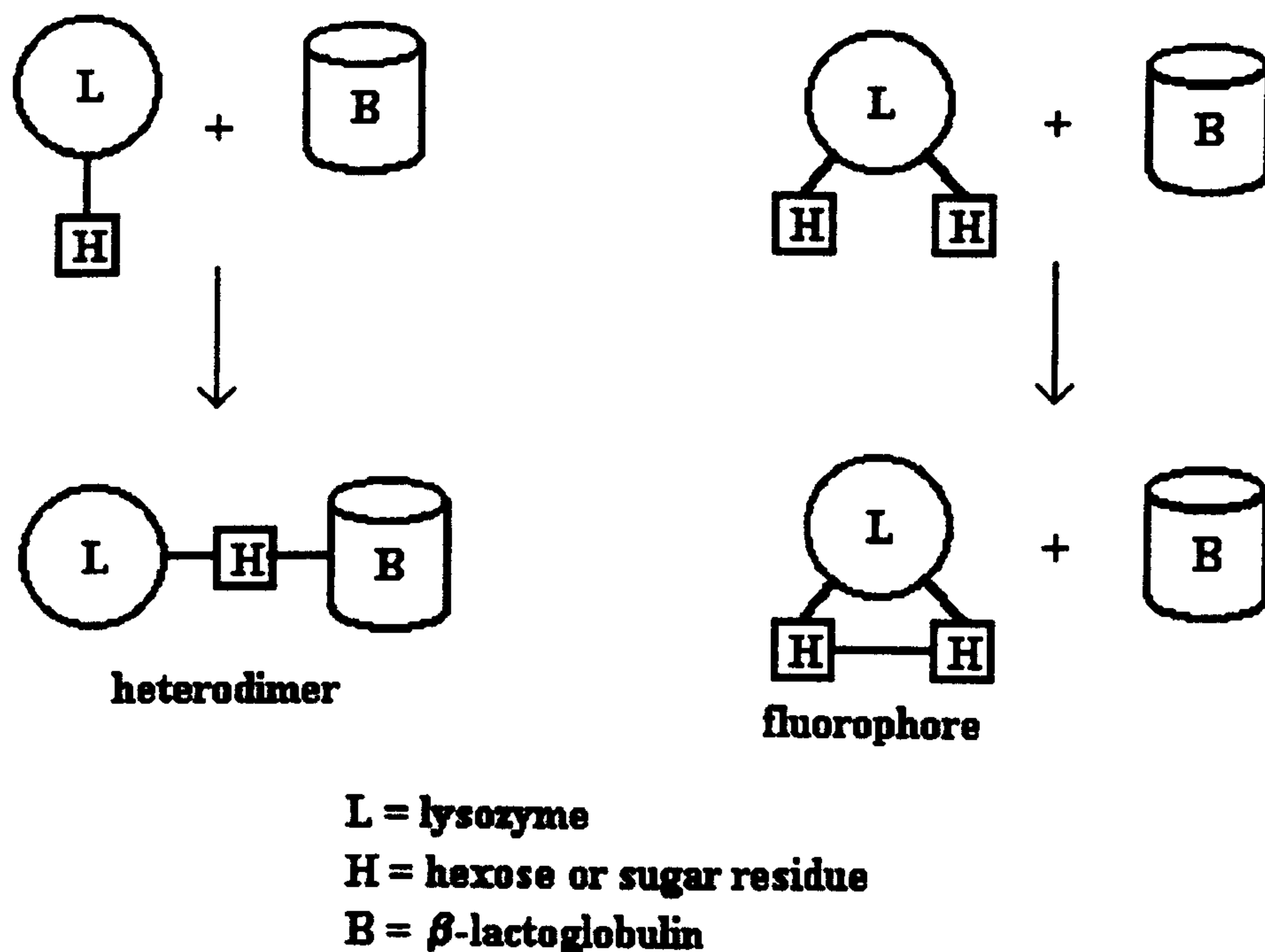


Fig. 5.5.3.1. A schematic diagram to illustrate that more extensively fructated lysozyme favours development of fluorescence rather than cross-linking with native  $\beta$ -lactoglobulin. Note that the diagram is speculative; currently the number of moles of incorporated fructose per mole of protein at which development of fluorescence is preferred over cross-linking, is unknown; also, two covalently-linked sugar residues on a protein do not necessarily form a fluorophore.

Sensi *et al* (1989) showed that *in vitro* glycated, powdered human glomerular basement membrane (10 days, 0.5M glucose, phosphate buffer) trapped more insulin, albumin, IgG and fibrinogen than the non-glycated control [181]. They

proposed that such trapping *in vivo* could exacerbate diabetic nephropathy. Indeed it would explain the increased deposition of albumin and fibronectin in diabetic kidney [191 and 208]. The *in vitro* studies carried out by Sensi *et al* (1989) have described glycated collagen trapping serum proteins [181]. The re-incubation experiments reported in this thesis are model systems that show that glycated proteins will form cross-links with other native proteins long after the removal of the monosaccharide. The results lend weight to the theory that the deposition of serum proteins onto the proteins of structural tissues, such as glomerular basement membrane, is via AGE-cross-links. Such AGE-cross-linking of serum proteins to structural proteins can be initiated by glycation of either or both of these two types of protein.

The 'minimally fructated' sample (Figs. 5.4.3.5. to 5.4.3.7.) indicates that the proportion of glycated protein in relation to the native form need only be small for AGEs to be formed and detected. The low concentrations of fructose found in human circulation combined with the non-detection of fructated protein, has led to the general assumption that dietary fructose has an insignificant effect on deterioration of health [209]. The results in this thesis show that both short exposure times of protein to high concentrations of fructose and long exposure times to low concentrations of fructose can, after removal of the free sugar, form AGEs. The results indicate that dietary fructose could *in vivo* fructate enough protein to contribute to the development of diabetic secondary complications and thus also to the same diseases that occur in old age.



## 5.6. Summary

1. Fructation of the proteins lysozyme,  $\beta$ -lactoglobulin, and BSA in isolation, gives rise to dimers and trimers in the case of the first two proteins and a 72kD protein in the third.
2. Fructation of lysozyme and  $\beta$ -lactoglobulin together produces a 32kD heterodimer.
3. Formation of cross-linked and fluorescent AGEs is inhibited by fructation in HEPES buffer, or by inclusion of aminoguanidine or DTPA in phosphate buffer. The inhibition indicates a role for carbonyl intermediates and metal catalysed oxidation in AGE formation.
4. The incomplete inhibition of AGE formation by aminoguanidine and DTPA indicates that some routes to AGE formation are independent of carbonyl intermediates and metal catalysed oxidation. The inhibitors also suggest different routes to formation of some non-fluorescent, cross-linked and fluorescent AGEs.
5. Lysozyme that has been minimally fructated (12 hours, 5mM fructose, 4°C in bicarbonate buffer) produces a heterodimer with  $\beta$ -lactoglobulin upon sugar-free re-incubation. Thus it is possible that even the low concentrations of serum fructose from the diet could cause significant levels of glycation *in vivo*, contributing to the progression of diabetic secondary complications and ageing.

6. The post-Amadori or post-Heyns production of non-fluorescent, cross-linked and fluorescent AGEs may be independent, although they may share common post-Amadori or post-Heyns intermediates.



## 6. Overall Discussion

The purpose of this chapter is to provide an overview of the results presented in this thesis, to correlate those results and discuss the contribution of fructose to the Maillard reaction *in vivo*. It is also an opportunity to discuss the contribution of fructation to progression of diabetic secondary complications and the same diseases that occur as a result of old age. The similarity of fructose and glucose chemistry makes it difficult to determine the relative contribution of each sugar to the formation of AGEs present in diabetic secondary complications. Conversely the slight difference in the chemistry of glucose and fructose means that the assays designed to detect glucation, underestimate fructation [209]. The underestimation of fructation is compounded by the low concentration of fructose in the blood. Dietary fructose is an important part of our diet; one of the questions to be raised is one of whether dietary fructose could significantly contribute to progression of diabetic secondary complications and the same diseases that occur in old age.

Before the turn of the century, fructose was only available in the western diet from natural sources, e.g. fruits and honey. At the turn of the century sucrose became widely used and its consumption has increased dramatically since then. Over the past 20 years the increased consumption of convenience foods (soft drinks, dairy products, canned, baked, and processed foods) has further increased our exposure to fructose. Initially sucrose was the predominant calorific sweetener in these foods, but this has been superseded by sweeter, high fructose corn syrups (HFCS). HFCS can contain up to 90% fructose by weight, but the majority (58%) that are used contain 55% fructose by weight (HFCS-55) [90 and 213]. Fructose is also used extensively in foods recommended for diabetics, this is because it has little effect on blood glucose concentration. The development of diabetic secondary complications is now widely appreciated to be a result of hyperglycaemia. What is

not quite so widely recognised is that other dietary sugars can also undergo the Maillard reaction, thus in theory contribute to diabetic long term complications and probably the same diseases that occur in the general population during old age. Our health throughout life is determined by our diet, dieticians have maintained for many years that a balanced diet is essential for a high quality of life. Fats are now widely recognised as important to the development of heart disease. I would like to see more research carried out on the role of monosaccharides in the development of such diseases, especially since diabetics suffer from them at an earlier age.

Fructose is also produced by the sorbitol pathway, which in certain tissues is an important route for metabolism of intracellular glucose during hyperglycaemia. The disruptive osmotic effect of sorbitol accumulation on crystallin fibres of the lens is one of the theories put forward to explain diabetic cataract. The end-product of the sorbitol pathway, fructose, has however been largely ignored. McPherson *et al* (1988) reported that 10 to 20% of hexose incorporated into human ocular lens formed the same derivative, upon assay, as fructose. The fructose that formed the fructated protein was suggested to originate from the sorbitol pathway; especially since the concentration of fructated protein was higher in diabetic lenses than those from non-diabetics. Interestingly the presence or lack of cataract did not effect the results; the small number of lenses assayed meant that no firm conclusions could be drawn, but the experiment does warrant further exploration with more lenses. If the results withstand statistical scrutiny they suggest that there is no connection between cataract formation and fructation [88]. It is difficult to justify the presence of fructose in the lens since it would be metabolically expensive for the endothelial cells of the lens to export fructose into the lens, unless it had reached a toxic concentration within the cells. Here toxicity is defined as osmotic pressure or formation of fructated protein or amino acids at a



rate that the cell is unable to cope with. It is however clear from McPherson *et al* (1988) that fructated protein is present in the lens; the fructose presumably being released from the epithelial layer of cells that surround the lens.

It has been known for a number of years that *in vitro* glycation by fructose proceeds in haemoglobin at a faster rate than glucose and similarly, that *in vitro* AGE-fluorescence develops at a faster rate during fructation than glucation [83 and 14]. The rate of *in vitro* incorporation of fructose or loss of free amino groups has, however, been reported to be equal or lower than that of glucose [88 and 14]. I have shown that *in vitro* fructose is incorporated into three different proteins at a faster rate than glucose and that this is reflected by their fluorescence. A higher rate of incorporation of fructose into protein would explain the faster development of AGEs upon fructation than glucation. Conversely, if glucose and fructose were incorporated at similar rates it would suggest that fructose produces more AGEs per mole of hexose than glucose. If more time had been available I would have liked to have further studied fructose incorporation into HSA. At 0.1M, fructose and glucose appear to be incorporated into HSA at very similar rates for periods of glycation up to 3 days (Fig. 3.4.1.1.). This result is possibly an error in the experiment but it would be worth checking; especially considering that the shorter incubation times are more physiological than a week of incubation using 0.1M hexose. I would also like to have investigated incorporation of hexose at concentrations much closer to those found *in vivo*.

The lack of assays for fructation has meant that fluorescence has been used as the standard method of its measurement throughout this thesis. Fluorescence is, however, a non-quantitative technique and caution must be used when comparing the results of experiments that use different proteins, buffers and sugar concentrations. With this in mind, when experiments are conducted under

identical conditions, the fluorescence is consistently higher upon fructation than glucation. Another consistent finding is that the fluorescence is inhibited by the metal chelator DTPA and the hydrazine aminoguanidine.

In chapter 3. I presented a histogram of the fluorescence of HSA and BSA developed over a week of fructation in various buffers (Fig. 3.4.2.1.). Current theories hold that post-Amadori and post-Heyns reactions are largely dependent on MCO. Bicarbonate buffer contains at least twice as much free metal than phosphate buffer, yet BSA developed much greater fluorescence in the latter. Delipidated, essentially globulin free, HSA did develop more fluorescence in bicarbonate, but it is likely that this protein has been oxidatively modified by its purification. There is therefore something in the phosphate buffer, other than free metal, affecting AGE formation. It is possible that inorganic phosphate catalyses Amadori or Heyns rearrangement. This opens an avenue of research into the effect of different anions on AGE formation. If more time had been available it would have been interesting to investigate the effect of different preparations of a variety of buffers on AGE formation.

The DNPH assay was developed to assess glycation by all sugars, specifically to quantify fructation. The assay does indicate enhanced rates of fructation and AGE formation when compared to glucation. Such a comparison does, however, need to be treated with caution because it relies on the assumption that products of glucation and fructation present the same proportion of reactive carbonyl groups. Since glucose and fructose themselves have very different percentages of acyclic aldehyde and ketone forms this assumption is likely to be wrong. Thus the above conclusion, that fructation and AGE formation from it proceeds at a faster rate than glucation, is drawn in combination with other assays of the Maillard reaction.



The investigation of the reaction of DNPH with intermediates and or products of the Maillard reaction was also useful for another reason. DNPH and aminoguanidine are both hydrazines that probably react with the same compounds. The production of DNPH positive material (protein-bound carbonyls) is inhibited by delipidation and chelation of free metal, as is fluorescence, whilst formation of Amadori product is unaffected. This indicates that the effect of metal catalysed oxidation and the action of lipid on the Maillard reaction is post-Amadori or post-Heyns and thus DNPH detects post-Amadori or post-Heyns compounds. The material detected by DNPH is not an advanced glycation end-products; as sugar-free re-incubation of fructated BSA results in a decrease in protein-bound carbonyl, whilst fluorescence increases as a function of time of re-incubation. Thus if the actions of DNPH and aminoguanidine are similar, a significant part of the action of aminoguanidine will be on this new class of post-Amadori or post-Heyns compounds. The inhibition of development of fluorescence by aminoguanidine indicates that the compounds detected by DNPH are intermediates or precursors in the formation of fluorescent AGEs. This conclusion is further supported by analysis of periodate, DNPH and fluorescence assays of protein glycated in the presence of aminoguanidine. Amadori product is unaffected or even promoted by the presence of aminoguanidine which is concomitant with it blocking after this point in the Maillard reaction, rather than, for example, on free sugar. Production of protein-bound carbonyl is partially inhibited and fluorescence is completely inhibited by aminoguanidine. If DNPH and aminoguanidine react with the same compounds, the partial inhibition of DNPH positive material can be explained by the chemistry of the hydrazones; the aminoguanidine hydrazone is probably hydrolysed by the acid used to solvate DNPH, the parent carbonyl is released and available for reaction with DNPH with some competition by aminoguanidine. Thus aminoguanidine blocks at the same

site detected by DNPH so preventing further AGE formation along this route, e.g. fluorescence. The lack of inhibition of Amadori product production by aminoguanidine is in agreement with the results reported by Requena *et al* (1993) and Fu *et al* (1994); the former article hypothesised that the main action of aminoguanidine was by sequestration of free monosaccharide; the latter article presents evidence similar to the contents of this thesis, i.e. blocking after Amadori product and hence its slight build up as a result [168 and 179].

Figure 5.4.2.2. illustrates that aminoguanidine only partially inhibits cross-linking yet the fluorescence has not increased (Fig. 5.4.2.3.). Two possible explanations for this result are; 1) there is a route to non-fluorescent Maillard cross-links that does not involve carbonyl formation. 2) the cross-links themselves, or their derivatives, present a carbonyl group and sequestration of this carbonyl group by aminoguanidine blocks its further part in the Maillard reaction, such as development of fluorescence.

The re-incubation of minimally fructated lysozyme with native  $\beta$ -lactoglobulin revealed that although cross-linking developed from lysozyme fructated for any amount of time, fluorescence only developed if the lysozyme was fructated for at least 3 hours (0.5M fructose at 37°C in phosphate buffer). This result indicates that cross-linking preceded fluorescence in the time scale of development of AGEs. It may be that some cross-links may themselves become fluorescent with time

Figure 6.1. is a modification of the schematic diagram of figure 5.5.2.1. produced using the conclusions of this thesis. The figure illustrates an area prior to production of protein-bound carbonyl groups that is dependent on metal catalysed oxidation, however, the results indicate that there is some AGE formation even when the metal content of the buffer is chelated or of very low concentration (as in HEPES). Metal ions chelated to compounds such as DTPA are still able to take part



in oxidation reactions; HEPES buffer is also not completely free of metal, nor is the water or labware used to make it. The low level of MCO could be responsible for AGE formation in HEPES or in the presence of DTPA, but an alternative explanation is that there could be routes to AGE formation that are independent of metal-catalysed oxidation.

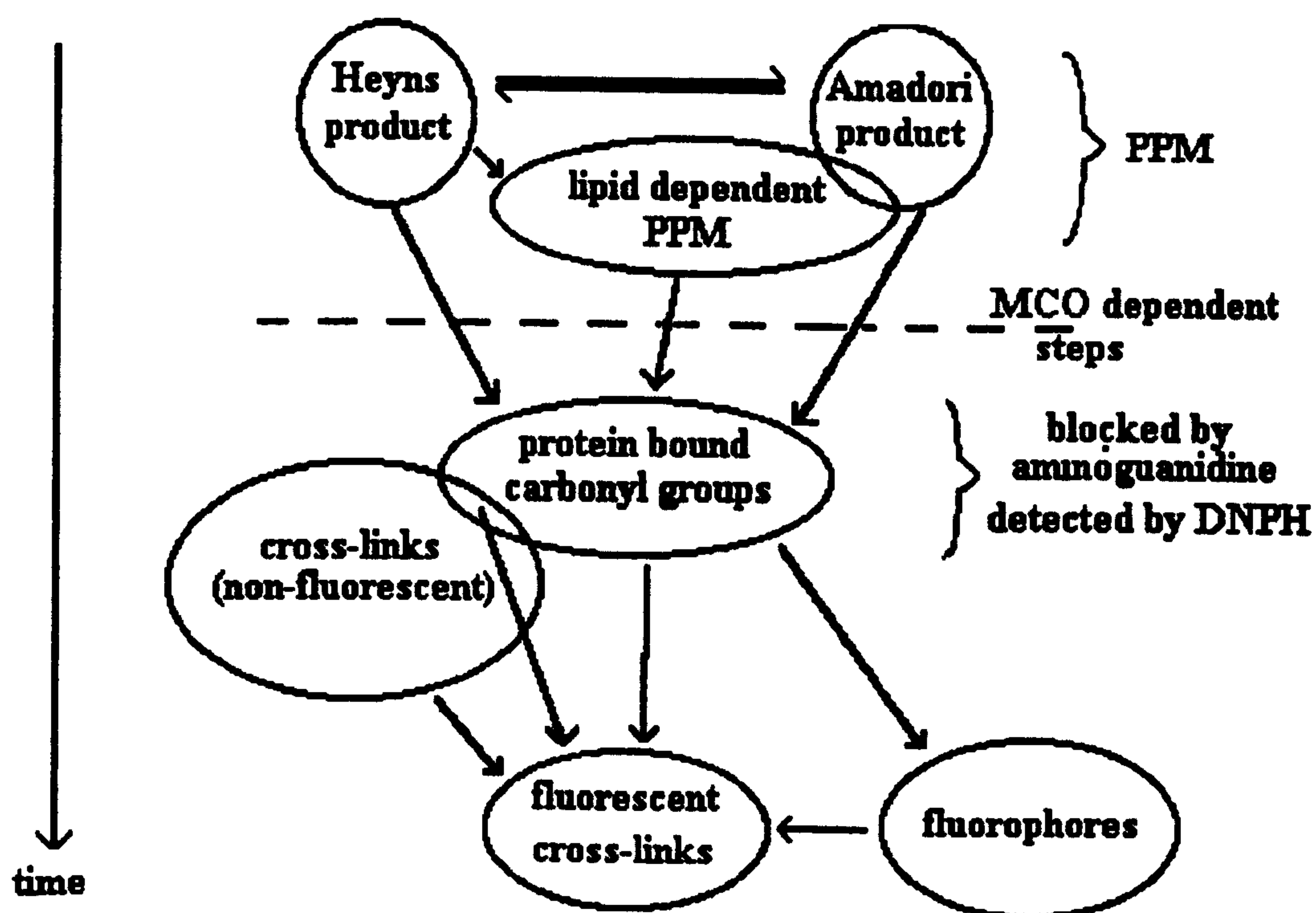


Fig. 6.1. Schematic diagram of post-Amadori or post-Heyns reactions. The boxed areas represent families or groups of compounds defined by their properties, thus overlapping boxes represent groups with both properties.

*In vivo* Maillard-cross-links are probably responsible for initiation and propagation of a multitude of degenerative diseases of old age and those associated with diabetic secondary complications. The majority of published studies of Maillard-cross-linking have concentrated on a protein in isolation (or a tissue rich in a single protein) such as collagen, this is because structural proteins such as collagen are associated with the majority of diabetic secondary complications. The mechanism and effects of deposition of serum proteins into basement membranes (other than

mere observation of deposition) has received little attention. Deposition of serum protein into a structural matrix bestows abnormal function to the basement membrane; e.g. immunoglobulins could initiate autoimmune disease [reviewed in 214]. The release of cytokines by macrophages attracted to AGE, or oxidatively, modified basement membranes could be responsible for the thickening of the membrane [215].

Chapter 5. of this thesis describes a model system for the study of cross-linking of a glycated protein with a different native protein upon incubation under conditions free of sugar. It provides a model to illustrate how a protein that is glycated in one part of the body can cross-link to another non-glycated protein somewhere else. This is especially important for dietary fructose which does not normally cause especially high concentrations of fructose in general circulation. Blood proteins are only normally exposed to elevated concentrations of dietary fructose in the blood between the small intestine and the liver (hepatic portal vein), for short times during absorption of an ingested meal. Thus proteins fructated in the blood of the hepatic portal vein are distributed throughout the body. Such glycated proteins are likely to have altered receptor mediated uptake and, as AGEs develop, become more resistant to enzymatic degradation by formation of intra-molecular cross-links. Fructated proteins in circulation have the potential to cross-link to structural proteins such as those in basement membranes. Such deposition would alter the properties of the basement membrane, as outlined in the paragraph above.

Chapter 5. also illustrates that the time of exposure of a protein to fructose need only be short, or at low concentration for 12 hours, for enough protein to be glycated such that cross-linking can be observed upon sugar-free re-incubation with a different native protein. Thus the model of minimal fructation mimics the



effect of dietary fructose *in vivo*; i.e. short periods of elevated fructose concentration (up to a few mM), and thus fructation, in the hepatic portal vein followed by a return to basal levels after absorption has finished, whereupon development of AGEs can occur unabated.

The results presented in this thesis do indicate a role for *in vivo* fructation derived from both dietary intake and the sorbitol pathway. The reactivity of fructose indicates that the sorbitol pathway is not a benign curiosity but is probably a serious contributor to diabetic secondary complications, thus is yet another reason for diabetics to maintain euglycaemia. The increased consumption of fructose in the west is probably contributing to the deterioration of health during old age. This deterioration could be reduced by avoidance of convenience foods and drinks; such advice is particularly relevant to diabetics who are already producing AGEs at elevated rates. The degree of contribution of fructose to diseases of old age and diabetic secondary complications is open to debate, it is likely to vary between individuals and their lifestyles. Lifestyle is a very important factor because the Maillard reaction is very slow in comparison to enzymatic reactions *in vivo*, the reaction and levels of its products are enhanced and or inhibited by other factors throughout the life of the subject. The body has the capacity for self repair, thus as long as this mechanism is not overstretched a healthy lifestyle will reduce any damage accumulated through life.

## 7. Suggestions for further study

The experiments investigating incorporation of hexose into protein could be taken further. The similar rates of incorporation of glucose and fructose into HSA over the first 3 days of glycation (Fig. 3.4.1.1.) need to be checked. The incorporation of physiological concentrations of sugars would help build up the picture of AGEs produced *in vivo*.

The effect of metal catalysed oxidation on development of AGEs could be investigated further using phosphate buffer prepared from a range of salts with different hydrations. The higher the hydration of the stock salt, the greater the contamination by free metal, thus a range of buffers could be prepared with equivalent physiological concentrations and pH but with an increasing amount of free metal contamination. This sort of experiment would eliminate variations in anion concentration, which may also affect the Maillard reaction.

A conclusive test of whether DNPH and aminoguanidine compete for carbonyl groups during the DNPH assay could be made by assaying for protein-bound carbonyl groups using different concentrations of DNPH. If the DNPH assay is detecting all the available carbonyl groups on a glycated protein, increasing the concentration of DNPH should have little effect on the results. Protein fructated in the presence of aminoguanidine could be assayed for protein-bound carbonyl groups using a DNPH assay with an increasing concentration range of DNPH. If the yeild of protein-bound carbonyl increases as a function of the DNPH concentration then the hypothesis; the aminoguanidine-protein hydrazone is hydrolysed by the acid used to solubilise DNPH and, subsequently, free aminoguanidine and DNPH compete for the same carbonyl groups; is true.



The experiments investigating the minimum conditions of fructose necessary to produce AGE formation could be taken further. Size exclusion chromatography combined with ultrafiltration is a quicker method for removal of free sugar than dialysis. If the time of exposure of protein to fructose is to be reduced further than reported in this thesis the time required to remove free sugar is crucial; this is because the Maillard reaction is continuing during this time. Quick removal of free sugar would ensure that the fructated protein had the least amount of time to form AGEs with itself and more glycated protein was available for cross-linking with a different protein.

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